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| (54) Title: THIN FILM HPMP MATRIX SYSTEMS AND METHODS FOR CONSTRUCTING AND DISPLAYING LIGANDS (57) Abstract <p>The invention relates to methods and systems of unhindered construction and display of tethered organic ligand molecules, and more particularly to preparation and use of thin film, substantially non-crosslinked hydrophilic polar multi-functionalized polymers (HPMPs) anchored to a variety of functionalized substrates so that the HPMP forms a thin film matrix layer providing a unique, highly hydrated, high dielectric environment equivalent to an aqueous solution, for affinity binding of Ligands (L) to Tagged Target Molecules (TTMs).</p> | | |

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SPECIFICATION

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**THIN FILM HPMP MATRIX SYSTEMS AND METHODS
FOR CONSTRUCTING AND DISPLAYING LIGANDS**

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BACKGROUND:

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This application relates to compositions and methods for synthesis and use of novel thin film hydrophilic molecular matrices for construction and display of ligands, especially bioactive molecules. More particularly, the invention relates to the use of thin film matrices of hydrophilic polar multi-functionalized polymers, such as carboxymethyl cellulose (CMC) or dextran, to which may be selectively attached and later ligated, a wide variety of ligands, organic and inorganic compounds of biochemical interest, for identification of the affinity and/or biochemical activity of the ligands with respect to selected tagged target molecules (TTMs), and for affinity purification or separation of TTMs from biochemical cocktails. By way of example of an important principal embodiment, the invention is directed to CMC or dextran matrix layers and their methods of preparation and use as environmental layers which are selectively chemically bonded (anchored) to a variety of substrates, and upon which selected organic molecules and mixtures thereof (ligands) may be assembled from synthons by known chemical processes, or pre-prepared and covalently bonded thereto, e.g., combinatorial libraries assembled from Aas, nucleotides, mono- or bicyclic ring compounds, sugars or the like, for display to determine their affinity to a variety of TTM's for a variety of purposes, e.g., purification, screening, amplification, isolation and the like.

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In modern molecular biology and pharmacology, it has been found that a variety of factors are involved in producing a specific biological response. On the molecular level receptor-mediated cellular processes, and

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1 binding of effector molecules may result from combinations
2 of hydrophobic, aromatic, charge transfer, salt bridging
3 and hydrogen bonding interactions. For productive binding
4 to occur it is vital that these forces and the groups
5 which mediate them be displayed in appropriate
6 conformations. A bioactive molecule may have agonist or
7 antagonist activity, and may be a vital component of
8 therapeutically useful compounds. Activity involves,
9 *inter alia*, the several interactions mentioned of a
10 biologically active molecule, a ligand, to a target
11 molecule. An example is where a small ligand effectively
12 blocks a larger ligand from binding to its target
13 molecule, a natural biological receptor, e.g., to prevent
14 the target from having an adverse effect on a tissue or
15 organism. Conversely, a ligand may "turn on" a target
16 molecule to engage in or initiate activity e.g.,
17 biochemical signal transduction.

18 These structure activity relationships are complex 3-
19 D puzzles complicated by the *in situ* environment and the
20 nature of the respective substituent groups of the ligand
21 and target. The target and ligand molecules in fact are
22 somewhat flexible, *inter alia* because of the manner in
23 which the constituent atoms thereof bond to themselves and
24 other molecules. For example, the flexure may be due in
25 part to hydrogen bonding of different degrees of strength
26 at different places along the molecules, and to the
27 rotation around covalent bonds in the ligand molecule.
28 Further, the isoelectric strength of the medium and the
29 environment of the molecules plays a very important role.
30 Steric hindrance by surfaces upon which small molecules
31 are displayed may totally mask significant binding
32 observable in solution. Display in proximity to surfaces,
33 or within gel type polymers, will be similarly effected by
34 the effective dielectric constant, changing the magnitude
35 of all binding forces, and grossly affecting conformation,
36 and therefore the "fit" between molecules.

37 Additionally, there are a wide range of degrees of
38 affinity "fit" between ligand and target, and likewise
39 between target and receptors, ranging from partial to

1 complete fit. There is a concomitant range of
2 effectiveness of a ligand to bind to target molecules, and
3 thereby trigger a natural receptor, block, or ameliorate
4 the adverse biological effects of the target on natural
5 receptors, or inhibit the activity of an enzyme.

6 Another aspect of the problem is the vast numbers of
7 potential candidate ligands considering the enormous
8 number of molecular, isomeric and polymeric formulas.
9 These numbers are increased by orders of magnitude when
10 considering the variety of steric configurations and range
11 of flexure. For example, considering the screening of
12 hexapeptide ligands for biological blocking affinity of
13 target molecules, there are some 64 million hexapeptides
14 constructable from all the combinations of the 20 D-amino
15 acids, an equal number for the 20 L-amino acids, and an
16 even greater number for non-natural amino acids. Where
17 the ligands are longer, the molecules have a greater
18 chance of adopting a number of different conformations and
19 thereby result in presentation of a number of different
20 possible affinity combinations.

21 The two commonly used approaches rely either on
22 solution interactions of the library ligand with a target,
23 or supporting the ligand on a solid phase. The advantages
24 of a supported library over solution interaction is the
25 ability to rapidly identify ligands by content
26 addressability, or to sequence the ligand or a surrogate
27 tag for the ligand after identification.

28 Accordingly, there have been created various sized
29 "libraries" of related organic molecules, i.e., a pre-
30 selected set of ligand variations, on different types of
31 solid supports. A variety of methods are known for
32 producing such solid-phase supported libraries of organic
33 molecules (ligands) as an aid in drug discovery. These
34 include the Selectide one peptide per bead approach, the
35 Affymax photolithographic approach, and the Arris PILOT
36 addressable array approaches. The PILOT approach is the
37 subject of co-pending SN 07/939,065, and the Selectide and
38 Affymax approaches are summarized in the background
39 thereof.

1 A major problem of potential library methods, is how
2 to "display" the combinatorial ligand constituents, the
3 concept of the term "display" including holding the ligand
4 on or securing it to the substrate. Many ligand molecules
5 have very low solubility in aqueous media, which is the
6 usual solvent or carrier for target molecules flowed
7 thereover during affinity screening. If the ligands are
8 displayed on glass, silicon or polystyrene surfaces, for
9 example, they can interact strongly with the surface on
10 which they are displayed, introducing an artifact into the
11 screening process. With the Affymax procedure, the bulk
12 effect of the rigid silica matrix not only restricts the
13 synthetic efficiency, but also introduces steric hindrance
14 barriers to access by the target acceptor molecules.

15 Access to displayed ligands by target molecules is
16 critical for success of the screening process. These
17 target molecules are often large proteins or nucleotides
18 which cannot easily diffuse into cross-linked gels.

19 It is particularly noteworthy that the Selectide
20 process uses cross-linked polyacrylamide and polystyrene
21 particles which permit reasonably efficient syntheses, but
22 the cross-linking prevents access of biological target
23 acceptors to any other than the surface molecules.

24 Accordingly, there is a need for a minimally
25 sterically hindered system and method for the assembly
26 and/or display of ligands for identification of binding
27 constituents of target molecules that does not introduce
28 unnatural steric artifacts or hindrances, yet is amenable
29 to rapid, addressable screening techniques, the goal being
30 highly sensitive biospecific ligand/target molecule
31 interaction screening, purification, isolation, recovery
32 and analysis.

34 THE INVENTION

36 OBJECTS

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38 Its an object of this invention to provide a
39 hydrophilic, polar multi-functionalized polymer (HPMP)

1 capable of forming an open, flexible, non-gel, essentially
2 non-crosslinked 3-D matrix layer which is selectably
3 anchorable to a substrate surface, and serves as a
4 "framework" for singly tethering preselected libraries of
5 MER_n ligands, and provides a highly accessible, flexible,
6 3-D display for free permeability of TTM's for highly
7 sensitive, amplified affinity binding.

8 It is another object of the invention to provide
9 methods for preparation, attachment and use of such HPMP
10 layers to a variety of substrates, preferably the polymer
11 discs of the PILOT addressable array systems.

12 It is another object of this invention to provide
13 systems and methods for amplifying displays of MER_n ligands
14 for affinity binding of TTM's.

15 It is another object of this invention to provide
16 thin matrix film ligand display systems to permit rapid
17 diffusion of unbound molecules from the matrix back into
18 the bulk solvent.

19 It is another object of this invention to provide a
20 ligand display system and method of identifying bound
21 targets, which system provides improved selectivity and
22 sensitivity.

23 It is another object of this invention to provide
24 systems and methods for displaying ligands for affinity
25 binding of targets under natural, hydrated conditions, and
26 which permits rapid elution of excess target and other
27 molecules.

28 It is another object of the invention to provide a
29 non-masking, freely permeable, open, 3-D matrix layer
30 providing a hydrated, high dielectric environment
31 substantially equivalent to a natural aqueous solution for
32 affinity binding of singly and permanently tethered
33 ligands to displaceable TTM's, which layer can be
34 selectively anchored to a wide variety of substrates, with
35 the anchoring being tuneable by adjusting the anchor
36 loading, and methods of preparation of the layer and use
37 thereof.

38 It is a further object of this invention to provide
39 HPMP's attached to surfaces suitable for the assembly of

1 (as shown in the lower half of Fig. 4) biomolecules from
2 sub-units or synthons, so that the ensemble is stable to
3 all synthesis and deprotection conditions.

4 Still other objects will be evident from the
5 specification, drawings and claims.

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7 **BRIEF DESCRIPTION OF THE DRAWINGS:**

8
9 The invention is illustrated in the drawings in
10 which:

11 Fig. 1 is a highly enlarged, schematic vertical
12 elevation of the HPMP matrix layer of this invention
13 stapled to a surface, having various mono-tethered ligands
14 and showing free access by TTM's;

15 Fig. 2 illustrates a presently preferred method of
16 attachment of the HPMP polymer via carboxyl activation and
17 covalent bonding to amino-functionalized substrate
18 surfaces;

19 Fig. 3 illustrates a first variation of the
20 attachment method of Fig. 2 in which masked functional
21 groups are incorporated into the HPMP;

22 Fig. 4A illustrates a second, converse method of
23 attachment of the HPMP, having amino-functionalized
24 groups, by addition to an activated carboxyl substrate
25 surface;

26 Fig. 4B shows the preferred amino-funtionalization
27 process for the polyethylene-dextran wink;

28 Fig. 5 is an HPLC chromatogram of removal of an
29 endorphin nonapeptide assembled on an HPMP wink of this
30 nonapeptide invention;

31 Fig. 6 illustrates the uptake of radioactivity from
32 ¹²⁵I labelled streptavidin on an HPQ ligand (which mimics
33 biotin) using the HPMP system of this invention;

34 Fig. 7 is a transmission spectrogram comparing a
35 bare, underutilized polyethylene wink with a carboxylated
36 wink;

37 Fig. 8 is an absorption spectrogram of an amino
38 functionalized polyethylene wink;

39 Fig. 9 is an absorption spectrogram of a dextran

1 loaded wink;

2 Fig. 10 shows a combined screening method where pairs
3 of known position 1 and position 2 amino acids in
4 hexapeptide are screened for relative bioactivity to
5 radio-labeled β -endorphin monoclonal antibody 3E7 as
6 determined by scintillation count;

7 Fig. 11 shows the results of sorting and screening
8 individual ones of the X-X-X-X-known dipeptide
9 alternatives of Fig. 10, as ligands, which exhibit the
10 highest affinity binding activity with 3E7 and their
11 resulting bioactivity (affinity binding) to the 3E7; and

12 Fig. 12 shows the relative bioactivity to 3E7 of a
13 hexapeptide sequence where YG is at the N-terminus
14 (positions 1 and 2) and the pair of alternatives shown in
15 the array of the figure at positions 3 and 4.

16 SUMMARY

17

18 The invention relates to methods and systems of non-
19 sterically hindered display of organic molecules, and more
20 particularly to preparation and use of substantially non-
21 crosslinked hydrophilic polar multi-functionalized
22 polymers (HPMPs) anchored to a variety of functionalized
23 substrates so that the HPMP forms a thin film non-masking
24 layer providing a unique environment substantially
25 equivalent to an aqueous solution, for affinity binding of
26 ligands (L) to Tagged Target Molecules (TTMs). Ligands,
27 and especially MER_n ligand libraries, are singly tethered
28 to the HPMP by a "permanent" strong covalent bond so that
29 subsequent displacement of the TTM does not also displace
30 the ligand from the HPMP.

31 The HPMP layer environment is a high accessible (to
32 the TTMs), flexible, 3-D display of the singly tethered
33 ligands providing free permeability therein of the TTMs
34 for affinity binding. The 3-D nature of the HPMP layer
35 provides a highly efficient interaction, between Ls and
36 TTMs, and thereby enhanced binding. Compared to flat
37 surface support-bound display methods, the 3-D matrix of
38 this invention permits a larger number of MER_n s to be
39 displayed for a given surface area, providing effective

1 amplification of the assay signals. Unlike cross-linked
2 thick gels, the open nature of the HPMP matrix and its
3 limited thin film depth permits rapid elution of excess
4 unbound TTMs and other molecules. The matrix results in
5 a highly hydrated, high dielectric environment similar to
6 an aqueous solution. Importantly, the HPMP matrix layer,
7 while providing amplification, is also non-masking, i.e.,
8 it is essentially transparent to detection methods. The
9 combination of the amplification and non-masking
10 properties results in a very significant, many-fold
11 increase in sensitivity of screening assays.

12 The displayed organic molecules and mixtures thereof,
13 herein broadly called ligands, include but are not limited
14 to, libraries assembled from synthons, such as AA's,
15 nucleotides, mono or bicyclic ring compounds, sugars, most
16 functionalizable organic moieties, and combinations
17 thereof. Other examples of the use of the system includes
18 use with biotin, antihistamines, benzodiazapines, and the
19 like, which bind biological receptors. The availability
20 of libraries of such diverse materials displayed on the
21 system of this invention will significantly expedite
22 discovery of new drugs.

23 The matrices and methods of the invention are more
24 specifically useful for optimal identification of a
25 binding constituent to any particular biologically
26 relevant protein, but may also be useful in a variety of
27 diagnostic and therapeutic applications. For example, the
28 system may be used for drug delivery. A drug may be
29 tethered by a cleavable linker to an HPMP matrix-coated
30 implant surface, and then under the effect of an
31 endogenous enzyme which acts on the linker, the drug is
32 slowly released, resulting in controlled drug delivery
33 over a long time period.

34 The system and method of this invention is compatible
35 with a wide range of organic molecules. It not only
36 facilitates their assembly, but also presents them in an
37 essentially 100% aqueous environment, and displays them so
38 that the biological acceptor molecule interacts with them
39 efficiently. It is a particular merit of this invention

1 that the nature of the molecular matrix cuts down
2 interactions between individual displayed ligand
3 molecules. This minimization of environmental effects
4 results in better synthetic efficiencies during assembly
5 of ligands from synthons than prior art systems.

6 The invention involves the addition of covalently-
7 modified long chain polysaccharides, such as CMC or
8 dextran, to functionalized substrate surfaces. The
9 substrate (SU) may be any bio-compatible, functionalizable
10 or pre-functionalized material capable of covalent bonding
11 to spacer arm anchor molecules. Examples of such
12 substrates include solid substrates (monolithic blocks),
13 membranes, films, laminates, spherical and irregular
14 particles, and woven or non-woven filtration materials
15 prepared, for example, from polyolefins, such as
16 polyethylene, polypropylene, halogenated polyolefins (such
17 as PVDF, PVC etc.); polystyrenes; polyacrylamides;
18 copolymers of the above co-polymers with the other
19 polymers; cellulose (including cottons, and other
20 natural and synthetic fibers), and inorganic materials
21 (including alumina, ceramics, silica, glass, and the
22 like). The currently preferred, best mode substrate is a
23 coarse polyethylene having 90-120 micron diameter pores.
24 One commercially available source of this material is
25 Porex Technologies of Fairburn, Georgia, USA.

26 While non-metallic substrates are preferred because
27 of the potential for unwanted adsorption effects, some
28 metals which can form covalent bonds with anchor molecules
29 to space all or part of the HPMP away from the surface can
30 be used. While noble metals such as gold cannot be used,
31 silicon and some metal oxides such as titanium oxide or
32 aluminum oxide may be employed.

33 The surface of the substrate may be functionalized in
34 a variety of ways to provide amino or carboxyl
35 functionalization sites for bonding thereto of the spacer
36 arm anchor molecules. For polyolefins, oxidation with
37 chromic acid provides a rapid and simple procedure.
38 Alternatively, other functionalization (e.g., via high
39 pressure reaction with oxalyl chloride, thionyl chloride,

1 plasma oxidation, or radical induced addition of acrylic
2 acid) may be provided. Halogenated materials (e.g.,
3 plastics) may be functionalized by base-catalyzed
4 elimination processes introducing double bonds, followed
5 by subsequent addition of amino-functionalized
6 derivatives. Silicas, aluminas, titanium oxides, ceramics
7 and silicons may be conveniently functionalized with any
8 of a variety of commercially available substituted silanes
9 (e.g., aminopropyltriethoxysilane). Hydroxylic compounds
10 (cellulosic membranes, filters, cottons, etc.) are simple
11 to derivatize via a variety of methods. They can be
12 directly carboxymethylated (e.g., with bromoacetic acid);
13 acylated directly with a protected amino acid (e.g., via
14 dimethylaminopyridine catalyzed carbodiimide coupling with
15 Tboe-glycine). A particularly advantageous method is
16 reaction with carbonyl diimidazole (or phosgene or
17 triphosgene), followed by reaction with a diamine or
18 monoprotected diamine to introduce amino functionalization
19 via a highly stable urethane linkage.

20 The HPMP matrix material may be any biocompatible,
21 substantially uncrosslinked, high molecular weight, highly
22 soluble polysaccharide, $[S]_n$. Particularly useful and the
23 currently preferred best mode are commercially available
24 high molecular weight dextrans (e.g., Pharmacia Dextran
25 T500), although a variety of other polysaccharides (e.g.,
26 carrageenans, and guaiac acid derivatives) are suitable
27 alternatives. Carboxymethyl- or amino-functionalized
28 polysaccharides such as cellulose are also suitable.

29 The degree of functionalization, expressed as
30 functional groups (FGs) per saccharide unit, $[S]$ ranges
31 from about 1:1 FG: $[S]_x$, to about 1:100 FG: $[S]_x$, i.e., x
32 ranges from 1 to 100 per functional group. The molecular
33 weight, MW, of the HPMP polymer may range from about 10K
34 (10,000) to about 10 MM (10 million), with an average
35 around 2 MM, where the polymeric unit is a saccharide.
36 For the typical polysaccharide HPMP of this invention $[S]_n$,
37 n ranges from about 500 to 50K. As noted below, the HPMP
38 strand is anchored in plural places to the substrate
39 surface, SU. In the anchored, folded or convoluted

1 condition, the HPMP forms a thin film layer on the order
2 of about 1000 Å thick (100nm), where $[S]_1 = 1 \text{ nm} = 10 \text{ Å}$.
3 Of course, other polymers will have somewhat different
4 lengths. The HPMP must have substantially no crosslinks
5 in order to maintain the matrix flexible, open and freely
6 permeable to the TTMs.

7 The attachment of the display matrix polymer strand
8 to the substrate surface may be performed by a variety of
9 procedures. It is important that the attachment chemistry
10 functions efficiently in aqueous media to form highly
11 stable chemical bonds. Single point attachment of an
12 HPMP, such as a dextran, to amino-functionalized surfaces
13 is obtained by reductive amination in the presence of
14 sodium cyanoborohydride, of the reducing end of the sugar
15 to an amino functionalized anchor. Alternately, there may
16 be direct attachment to an amino-functionalized substrate
17 surface. This method gives a low anchor density, i.e.,
18 number of anchors per cm^2 of substrate surface. We prefer
19 methods which form a plurality of attachment points (e.g.,
20 0.001% to 25% of available substrate surface
21 functionalized sites) that are stable during synthesis of
22 ligands and subsequent probings. Stable stapling is
23 needed so that what little glycosidic bond hydrolysis that
24 may occur between the HPMP and the spacer arm, happens
25 only during the more drastic chemical treatments involved
26 in subsequent synthesis of ligands (e.g., piperidine in
27 DMF, trifluoroacetic acid + ethane dithiol + thioanisole)
28 or during displacements of TTMs, so that little or none of
29 a displayed ligand library will be lost.

30 The most preferred attachment of the HPMP to the
31 substrate surface involves functionalizing the surface
32 with spacer arm anchor molecules and reacting them with
33 the HPMP by amide bonds. This process is termed
34 "stapling". If the surface bears pendant functional
35 carboxyl groups, e.g., terminal carboxyls on anchor
36 molecules, these must be coupled to amino-functionalized
37 HPMP's, e.g., aminated polysaccharides, by aminating the
38 HPMP before ligand tethering. Both the stapling linkage
39 and tether linkage functional groups can be provided

1 simultaneously by aminating the HPMP with sufficient amino
2 groups for both anchoring and for tethers for ligand
3 attachment. Conversely, if the support surface bears
4 pendant amino groups (on the surface itself or on
5 terminals of anchors) then attachment is achieved by
6 coupling to carboxyl functionalized HPMPs, e.g.,
7 carboxymethyl polysaccharides. Unreacted carboxyl groups
8 are then reactivated and coupled to mono-protected
9 bisamines to provide display sites for the ligands.

10 In another variation, both the masked amino and
11 carboxyl functional groups are incorporated on the
12 polysaccharide. Then, base treatment liberates carboxyl
13 groups for attachment, directly or indirectly, to amino
14 functionalized substrate surfaces or ligands.
15 Alternatively, acid treatment liberates amino groups on
16 the HPMP for attachment to surfaces or ligands that are
17 directly or indirectly carboxyl functionalized.

18 The chemistry used for polysaccharide-type HPMP
19 coupling (stapling and tethering) is essentially the same,
20 either pre-forming active ester derivatives with
21 carbodiimide reagents, and then adding the amino
22 component, or preforming activated species in the presence
23 of both amino and carboxy-components. Any particular
24 attachment can be "tuned", that is the amount degree or
25 density of anchors stapling the HPMP to the substrate
26 surface can be controlled, as well as the quantity, degree
27 or level of functionalization of the surface. Thus it is
28 possible to produce different proportions of
29 polysaccharide to surface attachment points (stapling)
30 compared to the loading (concentrating) ligand/library
31 display sites. The Detailed Description below gives
32 Examples of carboxy and amino substituted dextran-type
33 HPMPs at different levels of substitution, and different
34 relative attachment levels.

35 The use of spacer arm anchor molecules is preferred
36 over direct stapling of the HPMP to the substrate surface.
37 The spacer arms facilitate stapling of the HPMP to the
38 surface and, as noted above, permits control of anchor
39 density (degree of stapling). Where spacer arm anchors

1 are used, the spacer arm is covalently bonded adjacent one
2 end to the surface, as distinct from mere coordination or
3 adsorption to the surface, and the HPMP is covalently
4 bonded adjacent the other end of the anchor molecule. A
5 typical spacer arm molecule spaces the HPMP matrix layer
6 from about 15 to about 50 Å from the substrate surface.
7 A typical spacer may be any biocompatible
8 bifunctionalizable molecule that permits quantitative
9 control of attachment density to the substrate. Examples
10 of alternative spacers include: C₂-C₃₀ alkanes, polyethers
11 and combinations thereof, such as C₂-C₃₀ α, ω
12 diaminoalkanes, e.g. 1,3-diaminopropane and 1,6-
13 diaminohexane; a variety of peptides (e.g., oligomers of
14 beta-alanine, aminocaproic acid); polyglycol type
15 derivatives (such as Jeffamine ED-600 from Texaco, O,O' -
16 Bis (2-aminopropyl)-polyethylene glycol 500; and 2,2'-
17 (ethylenedioxy)-diethylamine from Fluka). The presently
18 preferred spacer is 2,2'-(ethylenedioxy)-diethylamine. A
19 typical density is 3x10¹⁵ anchors/cm² surface area. By the
20 derivitization process set forth herein, the variability
21 in numbers of anchors per cm² consistently can be
22 maintained to no more than 10%.

23 The preferred amino-functionalization process uses
24 the Fluka 2,2'-(ethylenedioxy)-diethylamine. Prepared
25 winks having an acid chloride surface are amino-
26 functionalized using the 2,2'-(ethylenedioxy)-
27 diethylamine. This provides a shorter spacer and allows
28 direct dextranization with subsequent amino-
29 functionalization of the dextran (for reaction with
30 tethers) by reaction with 1,3-diaminopropane.

31 As noted, it is important that there be substantially
32 no intra-HPMP crosslinks. The ligands are MER_n molecules
33 having potential affinity binding capability to selected
34 targets, where n of the ligand polymer (MER from polymer)
35 is in the range of from 2 to about 100 constructed
36 assembled monomeric units, such as polypeptides assembled
37 from Amino Acids (Aas). Typical ligands employed in the
38 system of this invention are MER_n ligand libraries
39 including polymers having MER-MER links of the following

1 types: amide; urethane; sulfonamid; thol; thioether;
2 ester; acrylic; and substituted amino (CONX) links. To
3 minimize steric hindrance and promote affinity binding,
4 the ligands are tethered to the HPMP through a single-
5 permanent strong covalent bond so that later displacement
6 of the mating affinity-bound TTM does not sever
7 (hydrolyze) the ligand from the HPMP.

8 The significance of covalent tethering should not be
9 underestimated. This permits the ligand library to be
10 used repeatedly, i.e., cycled repeatedly through the same
11 or different analyses. The bound libraries of this
12 invention are not destroyed or sacrificed with each
13 affinity screening or probe analysis. The HPMP bound
14 ligand libraries of this invention can be readied for
15 reassay by displacing a prior assay TTM by washing, e.g.,
16 with .1 M acid or alkali, or with 6 M urea, concentrated
17 guanidine Hcl, a denaturing agent or the like. The
18 library is then ready for another assay, since these TTM
19 displacement procedures do not sever the ligand/tether
20 bond. Since labeled TTMs are used, it is easy to check
21 that the prior assay TTMs have all been removed.

22 Examples of alternative tethers include: C₂-C₃₀
23 alkanes, polyethers and combinations thereof, such as C₂-
24 C₃₀ α, ω diaminoalkanes, e.g. 1,3-diaminopropane and 1,6-
25 diaminohexane; a variety of peptides (e.g., oligomers of
26 beta-alanine, aminocaproic acid); polyglycol type
27 derivatives (such as Jeffamine ED-600 from Texaco, O,O' -
28 Bis (2-aminopropyl)-polyethylene glycol 500; and 2,2'-
29 (ethylenedioxy)-diethylamine from Fluka). The presently
30 preferred tether is 1,3-diaminopropane.

31 It should be noted that the anchors and tethers may
32 be identical. Multi-functionalizing the HPMP followed by
33 using some of the functional groups for anchoring and some
34 for tethering, is one preferred approach. Thus, the HPMP
35 can be functionalized with amino or carboxyl groups, or
36 both (i.e., bi-functionalized) and a common anchor/tether
37 molecule, e.g., 1,6-diaminohexane, coupled to the HPMP to
38 form "linker" HPMP. The linker HPMP is then stapled to
39 the surface via only some of the linkers, and the ligands

1 attached on the remaining linkers, or vice versa, via
2 appropriate masking and reaction steps outlined above.

3 The nature of the HPMP layer as a hydrophillic thin
4 film is very important, since many ligands displayed as
5 part of libraries may have rapid dissociation of rates
6 from their targets. The thin film (a few 100's of Å to a
7 few 1000's of Å) permits rapid diffusion of an unbound TTM
8 to the bulk solvent outside the matrix before weakly bound
9 TTM's dissociate from their ligands. Thus, fast excess
10 TTM removal procedures compatible with the thin matrix
11 layer of this invention permit hitherto unobtainable
12 screening assays of fast offrate interactions.

13 The resulting surface-stapled HPMP layer is easily
14 and totally wettable. A preferred surface material is
15 polyethylene, in the form of a thin (ca. 1/8" thick) disc
16 (ca. diameter 1/4") called a wink. The HPMP layer is
17 totally transparent, and the HPMP layered wink exhibits a
18 wet sheen or reflectance, as distinct from the duller
19 plastic surface of the bare wink. When immersed in
20 aqueous solution the HPMP layered wink becomes
21 translucent.

22 Other important uses for HPMP matrices of this
23 invention include coating plates used in ELISA assays,
24 with covalent attachment to the matrix; for implanting
25 immunogenic peptides to raise antibodies for application
26 in immunotherapy, and for raising antibodies needed in
27 basic immunology research. The latter methods would be
28 superior to present methods, which use conjugation to
29 diphtheria toxins and other adjuvants which cause
30 unpleasant side-effects in humans and animals.

31

32 DETAILED DESCRIPTION OF THE BEST MODE:

33

34 The following detailed description illustrates the
35 invention by way of example, not by way of limitation of
36 the principles of the invention. This description will
37 clearly enable one skilled in the art to make and use the
38 invention, and describes several embodiments, adaptations,
39 variations, alternatives and uses of the invention,

1 including what we presently believe is the best mode of
2 carrying out the invention.

3 Fig. 1 is a schematic, highly magnified vertical
4 cross section of the HPMP matrix substrate system of this
5 invention. The substrate 1 is identified on the left as
6 SU. The surface 2 in this example is functionalized, and
7 spacer arm molecules 3 are covalently bonded at 4 to the
8 surface 1. This anchor layer is designated as A on the
9 left. The spacer arm anchors 3 are also covalently bound
10 to the HPMP strand 5 in a plurality of places 6, 7, 8.
11 The left hand strand is shown folded in a plurality of
12 folds F1 - F6 as shown. The right hand strand 9, shown
13 for clarity without ligands bonded by tethers is shown
14 convoluted, with anchors 10, 11, 12 more closely spaced in
15 a medial portion of the polymer chain. The precise
16 configuration is relatively intertwined, but the net
17 result is to create an open matrix layer, identified as
18 HPMP on the left. Various ligands, L, are tethered, T, to
19 the HPMP chain as shown, and the target molecules, TTM,
20 present in an aqueous solution (identified as AQ on the
21 left) freely permeate the highly hydrated matrix as shown.
22 The vertical height is not to scale in Fig. 1. The
23 anchors are typically of density 3×10^{15} anchors/cm², of
24 length about 15-50 Å, and the fully hydrated height of the
25 HPMP matrix layer is on the order of 200 - 2000 Å,
26 typically about 1000 Å.

27 The following examples set forth the best mode of the
28 steps of functionalizing a surface, stapling (anchoring)
29 a typical dextran-type HPMP, tethering ligands, contacting
30 with aqueous solution containing TTMs, and screening.
31

32 **Example 1. Oxidation of Polyethylene.** (Fig. 2, Step A)

33 Polyethylene discs (0.125" thick x 0.25" diameter)
34 having a pore size of 90-120 microns (Product # X-4906
35 from Porex Technologies, Fairburn, Georgia, USA) are
36 punched out of a coarse, porous polyethylene sheet 0.125
37 inches thick. These are called "winks", which, with their
38 high porosity are placed in a flask with an oxidation
39 reagent (CrO₃/H₂O/conc.H₂SO₄ at 56/80/56 w/w/w). The flask

1 is attached to a vacuum line and quickly evacuated and
2 purged. This process is repeated several times to ensure
3 that the inside core of each polyethylene winks is filled
4 with the oxidation reagent. Oxidation of the polyethylene
5 surfaces is preferably accomplished by raising the
6 oxidation reagent temperature to 85°C for 15 minutes.
7 After oxidation was complete, the winks were washed with
8 water, and dried overnight in vacuo, resulting in the
9 carboxyl wink of Fig. 2. The carboxyl functionality on a
10 polyethylene surface may be monitored by FT-IR
11 spectroscopy. Fig. 7 is a comparative transmission
12 spectrogram of a bare polyethylene wink (top curve) vs. a
13 carboxylated wink (lower curve) in which the 1720 cm⁻¹
14 wavenumber peak corresponds to, and provides positive
15 proof of, carboxylation.

16 **Example 2. Conversion to Acid Chloride.**

17 The above winks were placed in a round bottomed flask
18 under nitrogen and stirred with ethanol free chloroform (8
19 ml) and, preferably, thionyl chloride (2 ml), or,
20 alternately, oxalyl chloride in the same amount. After 1
21 hour the winks were washed with several portions of
22 chloroform (20 Ml) and dried in vacuo. This acid chloride
23 functionalized wink is shown on the upper left in Fig. 4.

24 The following Examples 3a, 3b, 4 and 5 show a variety
25 of embodiments of terminal amino-functionalized anchors
26 covalently bonded to discs as substrates.

27 **Example 3. Conversion of Acid Chloride to Amino-**
28 **functionalization. (Fig. 4A)**

29 a) As shown by step A in Fig. 4A, a first sample of
30 15 acid chloride winks were added to 1,6-diaminohexane (1
31 g) in methylene chloride (10 Ml). The mixture was shaken
32 overnight, then washed with DMF and methanol. Ninhydrin
33 and Fmoc-loading assays were used to quantify amino
34 functionalization of the resulting winks. Fig. 8 shows
35 the absorption spectrogram of an amino functionalized wink
36 manufactured by the process of Example 3(a).

37 b) As shown in step B of Fig. 4A, another sample of
38 the acid chloride winks were treated with polyethylene
39 glycol 500 diamine (Jeffamine ED 600) as above giving

1 similar loading of the resulting amide-bonded PEG wink
2 shown in Fig. 4A.

3 c) As shown schematically in Fig. 4A, step C, a
4 sample of 15 PEG amino-functionalized polyethylene winks
5 as prepared in Example 3 (b) were treated with succinic
6 anhydride (0.5 g), 1-hydroxybenzotriazole (0.3 g) and 4,4-
7 dimethylaminopyridine (12 mg) in DMF (10 mL). The
8 reaction was warmed to 60°C and shaken overnight. The
9 mixture was again warmed, shaken for 1 hour, then washed
10 with DMF, methanol and DMF. Seven of these winks were
11 washed with 1:1 DCM/DMF, then suspended in this mixture (2
12 mL) and treated with hydroxysuccinimide (NHS, 230 mg) and
13 di-isopropylcarbodiimide (DIPCDI, 0.32 mL). The reaction
14 mixture was shaken overnight, after which the winks were
15 washed with acetonitrile resulting in the NHS ester
16 functionalized (NHSEF) wink as shown in the lower half of
17 Fig. 4A, in an unblocked condition. The amino groups may
18 be all or partially blocked.

19 **Example 4. Introduction of Amino-functionalization via**
20 **Urethane. (Fig. 2)**

21 Another sample of several oxidized polyethylene winks
22 were placed in a round bottomed flask equipped with a
23 reflux condenser and stirred at 60°C in THF (10 mL) and 1M
24 borane in THF (20 mL), step B in Fig. 2. Stirring was
25 continued overnight, by which time reduction to alcohol
26 was complete. The reaction was quenched with water, and
27 the discs washed with water (3x), 1M HCl for 15 minutes,
28 water (3x), and then methanol (3x). The winks were dried
29 in vacuo overnight, then treated with 3 grams of carbonyl
30 diimidazole in 30 mL of DMF (step C in Fig. 2). Vacuum
31 was applied to the apparatus to degas the solution. After
32 3 hours, the discs were quickly washed with DMF 3x and
33 added to diaminoethane (6.5 g) in methylene chloride (35
34 mL). Again the mixture was degassed several times, and
35 then stirred overnight. The resulting urethane bonded
36 amino functionalized winks were washed with DMF (5x).

37
38 **Example 5. Addition of Beta-alanine. (Fig.**
39 **2, continued).**

1 Fmoc-beta-alanine (1.86 g), PyBOP (2.72 g), HOBt (0.8
2 g) dissolved in DMF (20 mL) was activated by the addition
3 of N-methylmorpholine (0.7 mL) and added to the amino
4 functionalized winks of Example 4, (see step D of Fig. 2).
5 Vacuum was applied twice to degas the mixture, which was
6 shaken overnight, treated with 20% piperidine in DMF for
7 20 minutes and washed with DMF (5x) and methanol (5x) and
8 dried resulting in the β Ala wink of Fig. 2.

9 The following two examples (6 and 7) show preparation
10 of a typical HPMP before anchoring to a substrate.

11 **Example 6. Low Carboxymethylated Dextran.**

12 1 g Dextran T500 (Pharmacia), bromoacetic acid (0.772
13 g), water (5mL) and 2M NaOH (5.6 Ml) were vortexed and
14 sonicated to form a clear viscous solution. This was left
15 for 20 hours. 1N HCl was added to give pH 3, and then 2
16 volumes of methanol added. A sticky white solid
17 precipitated and was collected by decanting the
18 supernatant. The precipitation was repeated and finally
19 dialyzed against water several times to give, on
20 lyophilization, the product as a very low density white
21 solid having the appearance of a soft spongy mass which is
22 easily separable into filamentary segments. ^1H NMR shows
23 ca. 1 carboxymethyl group per 4 sugars, considered a low
24 degree of carboxymethylation.

25 **Example 7. High Carboxymethylated Dextran.**

26 5 g Dextran T500 (Pharmacia), and NaOH (8g) were
27 dissolved in water (50 Ml), and bromoacetic acid (14.5
28 grams) was added. The mixture was shaken overnight. A
29 further 13.2 g bromoacetic acid and 16 grams of 50% NaOH
30 solution were added and the mixture shaken for a further
31 24 hours. 6M HCl (ca. 40 Ml) was added to bring to pH 2,
32 then the mixture was poured into 300 Ml of methanol. The
33 product separates as a white mass of the character
34 described above. A further 150 Ml of methanol was added
35 to complete precipitation. The precipitate was dissolved
36 in water, placed in dialysis tubes, and dialyzed against
37 4 changes of water. The product was obtained by
38 lyophilization as a white powder. ^1H NMR shows ca. 1
39 carboxyl group per sugar.

Example 8. HPMP (Dextranized) Polyethylene Substrate
Surface. (Fig. 2, steps E and F)

Method A: As outlined in Fig. 2, low carboxymethylated dextran of Example 6 (2g) and hydroxysuccinimide (NHS, 1 g) were treated with water soluble carbodiimide (EDC, 1.5 g) and the pH adjusted to 5 with 2N HCl. The beta-alanine winks produced in Example 5 were added, and the pH adjusted to 8 with 3.5 ml of 2M NaOH. The mixture was stirred overnight giving a gel like mixture. 1M NaOH (10 ml) was added and stirring continued for 35 minutes, to give a clear solution. Winks were washed with water (3x) and DMF (5x), resulting in the carboxyl dextran wink of Fig. 2. The dextran loading was determined using a total sugar assay (thymol-sulfuric acid).

Fig. 9 shows an absorption spectrogram of the carboxyl dextran wink of Fig. 2. The absorption peak occurs at a wavelength of approximately 510 nanometers. Excess amino-groups were then capped by acetylation with 0.3M acetic anhydride in DMF (20 ml) containing 0.03M dimethylaminopyridine. The winks were washed with DMF and methanol. As shown in step F of Fig. 2, a sample of eighty of these winks were suspended in water (10 ml), and hydroxysuccinimide (NHS, 0.4g) and EDC (water soluble carbodiimide, 0.6 grams) added. The mixture was sonicated and shaken for 15 minutes. A 3.5M solution of diaminopropane adjusted to pH 9 with c. HCl (2mL) was added. The reaction was stirred overnight. A further 0.4 Ml of the diaminopropane solution was added, and after a further 1 hour, the winks washed thoroughly with water, and DMF, resulting in the end product tether-loaded dextran wink shown at the bottom of Fig. 2.

33 **Example 9.** Amino-functionalized Dextran.

34 The high carboxymethyl substituted dextran of Example
35 7 (0.36g) was dissolved in water (5 ml) along with
36 hydroxysuccinimide (NHS, 0.28 g) and water soluble
37 carbodiimide (EDC, 1.31 g). The pH was adjusted to 5 with
38 2N HCl (50 μ L). After 15 minutes TboC-1, 6-diaminohexane
39 (0.52 g) was added, then 5 ml of water (pH of reaction =

1 8). After shaking at room temp for 30 minutes, the Ph had
2 fallen to 7. Then 2N NaOH (200 μ L) was added, and the
3 reaction shaken overnight. The product was dialyzed
4 repeatedly against water, and lyophilized. Since this
5 material had very low water solubility, it was not
6 characterized further, but treated directly with
7 trifluoroacetic acid (10 ml). Rapid carbon dioxide
8 evolution was observed, after 30 minutes anhydrous ether
9 was added (70 ml), and the precipitated product collected
10 by filtration, washed with ether, dried, redissolved in
11 water and lyophilized. The product was now highly water
12 soluble and NMR studies showed ca. 1 amino group per 2
13 sugar rings. This product (as shown in Fig. 4A) was
14 supplied for use in Example 10. The amino hexane side
15 chains function as linkers, either anchors or tethers.

16 **Example 10A. HPMP (Dextranized) Polyethylene Substrate**
17 **Surface. (Fig. 4A).**

18 **Method B:** A solution of the aminodextran (with side
19 chain linkers) from Example 9 was added to NHSEF winks of
20 Example 3 (c). The amino dextran solution was 150 mg in
21 1 ml water, with the pH adjusted to 7. The mixture was
22 sonicated then shaken for several days to staple the
23 dextran to the wink surfaces, i.e. some of the linker side
24 chains reacted to form anchors. The anchored winks were
25 washed with water, then methanol, and then dried to give
26 a substrate surface having an HPMP matrix layer with the
27 remaining unreacted amino-hexane linkers available as
28 tethers. The tether amino substitution level was 75
29 nanomoles. This is consistent with the NH_2 surface anchor
30 density of 70-80 nmole/wink. This process permits control
31 of the consistency of overall tether functionalization and
32 ligand loading from wink to wink to within 10% on a
33 thousand wink/batch scale. This end product is shown at
34 the bottom of Fig. 4A.

35 **Example 10B. Shortened Amino-Functionalization Process.**
36 **(Best Mode Fig. 4B)**

37 This is the preferred method for preparation of the
38 polyethylene-dextran HPMP surface. The process steps are
39 shown schematically in Fig. 4B. Acid chloride disks are

1 prepared following the procedures of Examples 1 and 2.
2 These disk were reacted with 2,2'-(ethylenedioxy)-
3 diethylamine in methylene chloride in the volume ratio of
4 1:10 v/v for 8 hours. Further treatment with high
5 carboxymethylated dextran (4.5 g) in the presence of water
6 soluble carbodiimide (EDC) (3.5 g) in 60 ml water at Ph 5
7 for 24 hours to "staple" the dextran to the wink providing
8 a substrate surface with a layer of carboxymethylated
9 dextran anchored thereto via the diamine spacers.
10 Attachment of 1,3-diaminopropane (1.5 g in 50 ml DMF) as
11 tethers to the carboxymethylated dextran was carried out
12 using 1,3-diisopropylcarbodiimide (1.2 g) and 1-
13 hydroxybenzotriazole (1.4 g) as coupling reagents.
14 Reaction at room temperature for 8 hours yielded the
15 amino-functionalized HPMP wink as shown at the bottom of
16 Fig. 4B.

17

18 **Example 11. Coupling via 2-Stage Reductive Amination**
19 **Procedure to Produce Thiol Substituted**
20 **Dextran.**

21 Dextran T500 (15 g) was dissolved in water (100 ml),
22 NaCl (15 g) added, then cysteamine hydrochloride (5.5 g).
23 The mixture was agitated vigorously to effect dissolution,
24 then 15M NaOH solution added to bring the pH to 9, and
25 finally sodium cyanoborohydride (5g) added. The extremely
26 viscous solution was mixed at 70°C for 2 days. The
27 solution was then cooled to room temperature, placed in a
28 dialysis bag, and dialyzed against several changes of
29 water. The solution was then treated with mercaptoethanol
30 (10 ml) and the pH adjusted to 8. After 2 hours the
31 material was repeatedly dialyzed against water; then
32 lyophilized to give mono-thiol-substituted dextran. Amino
33 substituted polyethylene discs (Example 3a) were treated
34 in DMF at 0°C with bromoacetic acid (0.14 g), pyridine
35 (0.05 ml) and dicyclohexylcarbodiimide (DCCI, 0.103 g).
36 After 1 hour reaction at room temperature, the
37 bromoacetylated surfaces were washed with DMF to produce
38 bromoacetylated winks. The thioated dextran solution (100
39 mg in 1 Ml water), was treated with sodium borohydride (10

1 mg for 1 hour) to reduce disulfide bridges. The excess
2 borohydride was destroyed by treating with acetone - 2
3 drops added, pH adjusted to 4, left 10 minutes, and the pH
4 then readjusted to 7. Then the bromoacetylated winks were
5 added to this thioated dextran solution. After shaking
6 for 1 day the discs were washed with water, methanol, and
7 dried to produce end product thio-anchored dextran winks
8 (not tether loaded).

9 **Example 12. Production of Dextrans Containing Masking**
10 **Functional Groups. (Fig. 3).**

11 This method allows precise control of loading of
12 tethers for attaching ligands. High carboxymethyl dextran
13 (Example 7, 0.36 g) was dissolved in water (7 ml) and
14 treated with hydroxysuccinimide (0.28 g) and EDC (0.31 g).
15 The pH was adjusted to 5. After mixing for 15 minutes, a
16 solution of TboC-1,3-diaminopropane (0.28 g, 2 equivs.)
17 and aminocaproic acid methyl ester hydrochloride (0.14 g,
18 1 equiv) in water (2 ml). After 5 minutes an additional
19 equal volume of 2N NaOH was added to maintain pH. The
20 reaction was shaken overnight, then dialyzed repeatedly
21 and lyophilized to give the diprotected product. NMR
22 studies show incorporation of the 2 functional groups in
23 the correct ratios. This masked bi-functional dextran
24 (MBD) product is shown at the top of Fig. 3. As shown in
25 step A, MBD dextran product was treated with 1N NaOH (10
26 ml) for 1 hour, the pH adjusted to 3, and dialyzed and
27 lyophilized as above described to give an amino-masked
28 carboxyl HPMP dextran (AMCD) derivative, as shown by NMR.

29 As shown in step B of Fig. 3 this AMCD HPMP was
30 coupled to amino-functionalized PE disc surfaces by the
31 process of step E of Fig. 2 described in Example 8.
32 Subsequent TFA treatment then liberated amino sites for
33 library synthesis. The end product tether loaded dextran
34 wink variation is shown at the bottom of Fig. 3.

35 **Example 13. Comparative Test - TFA Stability of Stapled**
36 **Dextranized Surfaces Compared to**
37 **Epichlorohydrin Bonding.**

38 Winks were dextranized in accord with the above
39 Examples 8 and 10 (Sample A). By way of comparative

1 tests, dextran was bonded to polyethylene surfaces using
2 epichlorohydrin (Sample B), as used by Pharmacia Biosensor
3 AB in connection with the BIAcore_{tm} surface plasmon
4 resonance biosensor systems (see Lofas, S. and Johnson,
5 B., A Novel Hydrogel Matrix on Gold Surfaces in Surface
6 Plasmon Resonance Sensors for Fast and Efficient Covalent
7 Immobilization of Ligands, J. Chem. Soc. Chem. Commun.
8 1990 pp. 1526-1528; also see WO 92/06380, WO 90/05295 and
9 WO 90/05305). Both samples were treated with TFA for
10 various time intervals up to 2 hours. The samples were
11 then analyzed by quantitative sugar loading assays. After
12 only 2 hours, around 90% of dextrans were lost from the
13 epichlorohydrin bonded surfaces, whereas only minor loss
14 (<10%) was detected from the wink surfaces stapled
15 according to the processes of this invention.

16 **Example 14A. HPMP Substrate**

17 The HPMP substrate was assembled on approximately 100
18 micron porous polyethylene winks. Using the preferred
19 oxidation method of Example 1 and the preparation scheme
20 outlined in Fig. 4B, consistent loading of 70-80 nmole
21 amino functional groups per disk was achieved. These
22 disks were subsequently used for peptide library synthesis
23 and display for screening.

24 **Example 14B. Peptide Synthesis on HPMP.**

25 A variety of peptide sequences were assembled on
26 tether loaded HPMP wink surfaces in accord with the
27 invention. Fig. 5 shows assembly of the octapeptide
28 YGGFLSGG- β Ala, the epitope of the 3E7 antibody, on stapled
29 dextran winks of Example 8. A hydroxymethylbenzoic acid
30 cleavable linker was first added to the tether, and then
31 the peptide chain assembled by an automated peptide
32 synthesizer (Millipore) by conventional peptide synthesis
33 chemistry. The peptide was treated with reagent R, a
34 standardized TFA cocktail, to remove side chain protective
35 groups, and then cleaved with .1 M NaOH for 1 hour. The
36 HPLC chromatogram of Fig. 5 shows the resulting extremely
37 sharp elution peak at 8.75 minutes, which demonstrates
38 recovery of highly pure peptide after cleaving. This
39 establishes covalent linkage for display via the tether on

1 tests, dextran was bonded to polyethylene surfaces using
2 epichlorohydrin (Sample B), as used by Pharmacia Biosensor
3 AB in connection with the BIAcore_{tm} surface plasmon
4 resonance biosensor systems (see Lofas, S. and Johnson,
5 B., A Novel Hydrogel Matrix on Gold Surfaces in Surface
6 Plasmon Resonance Sensors for Fast and Efficient Covalent
7 Immobilization of Ligands, J. Chem. Soc. Chem. Commun.
8 1990 pp. 1526-1528; also see WO 92/06380, WO 90/05295 and
9 WO 90/05305). Both samples were treated with TFA for
10 various time intervals up to 2 hours. The samples were
11 then analyzed by quantitative sugar loading assays. After
12 only 2 hours, around 90% of dextrans were lost from the
13 epichlorohydrin bonded surfaces, whereas only minor loss
14 (<10%) was detected from the wink surfaces stapled
15 according to the processes of this invention.

16 **Example 14A. HPMP Substrate**

17 The HPMP substrate was assembled on approximately 100
18 micron porous polyethylene winks. Using the preferred
19 oxidation method of Example 1 and the preparation scheme
20 outlined in Fig. 4B, consistent loading of 70-80 nmole
21 amino functional groups per disk was achieved. These
22 disks were subsequently used for peptide library synthesis
23 and display for screening.

24 **Example 14B. Peptide Synthesis on HPMP.**

25 A variety of peptide sequences were assembled on
26 tether loaded HPMP wink surfaces in accord with the
27 invention. Fig. 5 shows assembly of the octapeptide
28 YGGFLSGG- β Ala, the epitope of the 3E7 antibody, on stapled
29 dextran winks of Example 8. A hydroxymethylbenzoic acid
30 cleavable linker was first added to the tether, and then
31 the peptide chain assembled by an automated peptide
32 synthesizer (Millipore) by conventional peptide synthesis
33 chemistry. The peptide was treated with reagent R, a
34 standardized TFA cocktail, to remove side chain protective
35 groups, and then cleaved with .1 M NaOH for 1 hour. The
36 HPLC chromatogram of Fig. 5 shows the resulting extremely
37 sharp elution peak at 8.75 minutes, which demonstrates
38 recovery of highly pure peptide after cleaving. This
39 establishes covalent linkage for display via the tether on

1 the HPMP dextran, and that ligand assembly had
 2 successfully occurred in situ on the HPMP. The relative
 3 purity of the recovered peptide shows the assembly
 4 sequencing was synchronous and complete at all the sites
 5 on the HPMP. Additional experimental work established the
 6 use of other cleavable linkers, such as the PAL linker
 7 (Millipore) which was cleaved with TFA.

8 **Example 15. Signal to Noise Ratio.**

9 Amino-functionalized tether-loaded winks of Example
 10 8 had the ligand HPQFVSGG- β Ala (herein HPQ) assembled
 11 thereon by the process as in Example 14B. This ligand
 12 exhibits weak binding with Streptavidin, in that
 13 micromolar quantities need be present for binding to be
 14 detected as compared to the strong binding of YGGFLSGG-
 15 β Ala of Example 14B in which only nanomolar quantities
 16 need be present to exhibit uptake of 3E7 anti-endorphin
 17 monoclonal antibody.

18 The peptide loading of the winks was 50 nmoles/wink.
 19 The winks were suspended in 150 μ L of phosphate buffered
 20 saline, and various concentrations of 125 I-radiolabeled
 21 Streptavidin were added and incubated for ca. 2 hours.
 22 The winks were washed and counted on a gamma counter to
 23 determine binding saturation.

24 **TABLE I HPQ Winks Uptake**

| 26 TEST | 27 Amount | 28 Counts |
|---------|---|-----------|
| 29 per | 10 ⁶ | Minute x |
| | 125 I-Streptavidin | |
| 30 1 | 10 μ L | .32 - .5 |
| 31 2a | 10 μ L + 10-fold cold | .32* |
| 32 2b | 100 μ L | 3.2 |
| 33 3 | 100 μ L + 10-fold cold | 2.1-2.8* |
| 34 4 | 100 μ L + 100-fold cold | .69** |
| 35 * | Multiply by 10 for conversion to 10-fold cold corrected counts. | |
| 36 ** | Multiply by 100 for conversion to 100-fold cold corrected counts. | |
| 37 | 10 μ L of 125 I-Streptavidin exhibits 750,000 cpm and | |
| 38 | equals .1 μ g, about 1.6 Pm Streptavidin. Test #1 shows a | |
| 39 | range for several runs (5) of 320,000 to 500,000 counts | |
| 40 | per minute, an excellent uptake in the range of 42-67%. | |

Standard practice is to use non-radiolabeled Streptavidin, called "cold" solution, to dilute the radiolabeled sample. This permits higher concentrations to be assayed without massive amounts of radioactive material being employed. Thus, Tests 2a and 2b can be directly compared, 2b being a full concentration radiolabeled sample. Since the 2b counts are equivalent to the dilution multiple counts of 2a, the cold dilution sample 2a multiplied by the 10-fold factor is accurately representative of a non-diluted test. That is, Test 2a/2b confirms the validity of using the "X-fold cold" multiplying factor for ligands on the HPMP of this invention.

The value of 69×10^6 cpm in Test 4 corresponds to about 147 pmoles Streptavidin bound to the wink. AA analysis showed 10-20 nmoles of peptide on each wink. This series of tests represents "signal" strength.

A second series of tests of non-ligand-loaded but amino-functionalized (50 nmoles) winks was run to determine background "noise". These non-loaded winks were incubated in a vial of 150 μ L of the saline buffer, and 10 μ L 125 I-Streptavidin was added and incubated. After incubation the supernatant solution was pipetted off and the winks washed by various techniques. Table II shows the results.

TABLE II Unloaded Wink Washing

| TEST | Amino-functionalized Non-Ligand-Loaded Winks | CPM | |
|------|--|-------------------|--------------------|
| | | Supernatant | Bound |
| | | (Unbound) | Wink |
| 5 | Vortex Washing 5x .7 ml buffer | 680,000 | 6,957 |
| 6 | Vacuum Washing 5x .7 ml buffer | 660,000 | 3,824 |
| 7 | Centrifuge 3 min 13k | 690,000 | 10,117 |
| 8 | Vacuum Washing 25 ml | 750,000 | 350 av 3 runs |
| 9 | Vacuum Washing | 7.5×10^6 | 2,100 av 2 runs |

These results show an extremely low background count noise level, which means that even the very large

1 Streptavidin tetramer molecule of 60,000 Daltons MW can be
2 easily washed out of the convoluted HPMP matrix. Thus,
3 the incidence of the Streptavidin becoming caged in the
4 matrix is very low, with the result that false binding is
5 not an appreciable effect. In addition the S/N ratio is
6 very high. In Test 9, for example, the unbound is about
7 7.5×10^6 counts; from Test 2b above one would expect 3.2
8 $\times 10^6$ bound. But only 2100 counts were registered which
9 shows the S/N ratio was over 1500 bound/unbound residual
10 in the HPMP matrix.

11 Test 7 shows that only a thin film of solvent plus
12 solute is tightly bound as a hydration layer for the HPMP,
13 allowing assay of extremely fast offrate interactions.
14 Even in the absence of washing, an equilibrium enrichment
15 of 100:1 of the labeled Streptavidin would be detected.

16 **Example 15b. Library Screening.**

17 Simultaneous screening of large mixtures of candidate
18 compounds for bioactivity (affinity binding) to target
19 molecules requires a system which has a very high signal
20 to noise ratio since each single compound is present in
21 very low concentration. An example of a well-described
22 antibody test system which has been investigated by many
23 different methods is shown using the PILOT system in an 8
24 by 8 array employing the HPMP winks of the present
25 invention.

26 The libraries are prepared by coupling to each amino-
27 functionalized tether-loaded wink a mixture of 16 amino
28 acids at concentrations yielding equal incorporation of
29 each amino acid as described in our copending case
30 PCT/US/93 08267. At two chosen positions in the peptide
31 chain, pairs of these amino acids are coupled in rows and
32 columns to form the PILOT array. The resulting first
33 iteration library is washed with a solution containing
34 target molecules which preferentially bond by affinity
35 only to congruent hexapeptides in the library displayed in
36 the HPMP layer. The data shown in Table III and Figs 10-
37 12 is for the β -endorphin monoclonal antibody 3E7
38 (recognition sequence YGGFL) radio-labeled with sulfur
39 labeling reagent (Amersham) as the target molecule. The

1 labeled target protein that binds to the ligands of each
2 wink was determined by displacing the target protein with
3 washes of 0.1 N hydrochloric acid and guanidine
4 hydrochloride, and then counting the combined washes in a
5 scintillation counter.

6 In the first iteration library array, as shown in
7 Table III and Fig. 10, each wink has a mixture of hexamers
8 attached. Each hexamer on any wink has either of two
9 amino acids at the N-terminus, the first or A₁ position,
10 and either of two amino acids at position 2 (the A₂
11 position). Positions 3 through 6 (C-terminus) comprise a
12 random mixture of approximately 65,000 peptides made from
13 coupling the mixture of 16 amino acids four times. The
14 hexapeptide is generally identified as A₁ or 2A₃ or 4 XXXX.
15 The labeled target protein was applied to this library
16 array, incubated to allow specific binding to occur, and
17 unbound protein was washed away. The bound protein was
18 eluted and measured by scintillation counting. See Table
19 III and Fig. 10 and the preferred AAXXXX sequences
20 identified by their address in the array.

21 In order to further define (sort or characterize) the
22 N-terminal amino acid sequence, the peptides from the
23 areas of highest binding were repared on winks as a 2nd
24 iteration library having at each address a single known
25 two-amino acid sequence at the N-terminus. That is, only
26 one of the four combinations of A₁A₂XXXX, that were
27 together present at a single address in the first
28 iteration library, is present in each address. This
29 library of winks was assayed (Fig. 11) and the single best
30 N-terminal dimer sequence was determined to be YG. For
31 the third iteration YG was chosen as the N-terminus for
32 each wink, with the paired known array at positions 3 and
33 4 (Fig. 12); positions 5 and 6 are from two rounds of
34 coupling the random mixture of 16 amino acids. This Figure
35 12 shows a number of potential hexapeptide "drug"
36 candidates (or families) which can be specifically
37 characterized in subsequent iterative screening.
38 Likewise, characterization (identification of best AAs) in
39 positions 5 and 6 via iterative screening can then be

1 done.

2

3

TABLE III: SCINTILLATION COUNTS
OF B-ENDORPHIN MONOCLONAL
ANTIBODY 3E7 BIOACTIVITY

4

5

6

7

8

Position 1 Position 2

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42

| | Y,F | D,E | K,R | Nle,V | G,DAla | A,S | H,Q | P,DNap |
|---------|-------|-------|-------|-------|--------|-------|-------|--------|
| Y, F | 5420 | 544 | 1852 | 1387 | 3E+05 | 20581 | 899.6 | 2828 |
| D, E | 410 | 189.4 | 302 | 214.6 | 248 | 187.2 | 193.8 | 324.4 |
| K, R | 1647 | 233.2 | 1416 | 10.10 | 736 | 699.6 | 933 | 1030 |
| Nle, V | 2119 | 206.2 | 1021 | 585.2 | 351.2 | 375 | 394.6 | 1524 |
| G, DAla | 630 | 199.8 | 459.6 | 455.4 | 222.2 | 146.4 | 277.6 | 319.2 |
| A, S | 827.2 | 201.8 | 448.6 | 260.6 | 204.4 | 214.8 | 239.6 | 466.2 |
| H, Q | 1264 | 234 | 596.2 | 447.6 | 185 | 239.2 | 271.2 | 678 |
| P, DNaP | 4636 | 306.8 | 2318 | 1893 | 680 | 766.8 | 952.2 | 2629 |

Total cpm counted 1,722,258 *DNap =D-3 (2-naphthyl)-alanine

total cmp added 4,500,000

Numerous binding sequences are identified by this system. Fig. 12 shows that the major determinant suggested is the presence of an aromatic amino acid in either position 3 or position 4. These data correlate well with the data produced by Elisa measurement on mixtures of peptides free in solution (Houghten et al, Gene, 128 (1993) 71-76). The outstanding aspect of this data is the high signal from positive affinity binding as compared to the low background of non-specific binding. This high signal to noise ratio is an expression of the very high sensitivity and amplification of the thin film HPMP matrix of this invention. In turn this allows the identification of numerous weak binding variations which could not otherwise be identifiable. This is vital as in some instances only weak ligand-TTM binding may be possible, and thus drugs can be developed in such difficult-to-bind cases.

1 **Example 16. Quantitative Sensitivity.**

2
3 The HPQ ligand was assembled as above on tethered
4 dextran HPMP stapled winks of Example 8 but without a
5 cleavable linker, and Reagent R used to remove side chain
6 blockers. Several different loading samples were
7 prepared: one with zero (no HPQ); two at 50 nmoles; and
8 one at 80 nmoles. These HPQ ligands loaded on HPMP
9 tethers were incubated for several hours to progressively
10 increasing amounts of ^{125}I labeled Streptavidin, as a
11 sample TTM, in buffered saline solution. The winks were
12 removed, vacuum washed in the buffered saline, and the
13 bound Streptavidin counted. Table IV shows the data and
14 Fig. 6 graphically shows the results as smoothed curves.
15

16 **Table IV HPQ Saturation in CPM $\times 10^6$**

17

| Test # | HPQ Octapeptide Loading on HPMP | | | | | | | |
|--------|---------------------------------|---------|-------------|---------|-------------|---------|------------|---------|
| | 50 nmoles • | | 80 nmoles ▲ | | 50 nmoles ▲ | | Unloaded • | |
| | Bound | Total | Bound | Total | Bound | Total | Bound | Total |
| 10 | 0.500 | 0.775 | 0.042 | 1.140 | 0.013 | 0.650 | 0.000 | 0.500 |
| 11 | 3.200 | 7.750 | 0.775 | 11.690 | 1.260 | 6.700 | 0.001 | 0.650 |
| 12 | 9.800 | 23.250 | 0.719 | 6.456 | 0.559 | 4.520 | 0.002 | 6.250 |
| 13 | 24.500 | 77.500 | 14.392 | 64.160 | 9.919 | 45.200 | 0.005 | 62.500 |
| 14 | 69.000 | 775.000 | 94.361 | 624.800 | 61.052 | 452.000 | 0.005 | 625.000 |

25
26

27 Fig. 6 shows the data in a graph form. The curve of the
28 solid triangle data points represents an 80 nmoles-loaded
29 peptide on the HPMP. The solid circle and triangle are 50
30 nmoles loadings on two slightly different HPMP lots. The
31 comparison non-loaded amino-functionalized winks are
32 represented by the solid square symbols; they do not even
33 show on the abscissa of the graph, i.e., the background
34 noise curve is essentially flat. The 80 nmoles curve
35 peaks at about $130\text{--}140 \times 10^6$ CPM. The results show
36 essentially linear uptake through about 50-80 million CPM.
37 This means that the HPMP system behaves exactly as it
38 would be predicted, had the measurements been carried out
39 with untethered molecules in solution. Also the HPMP

1 system of the invention can be used quantitatively; that
 2 is, count readings (intensity) can be quantitatively
 3 correlated to amounts of bound TTMs. The high slope and
 4 total amount of bound counts indicates high sensitivity of
 5 the method.

6 **Example 17. Fast Off-Rate Analyses.**

7 Over washing can remove specifically bound ligands if
 8 these ligands have a fast off-rate. The more extensive
 9 the washing the more signal is removed. In cases of fast
 10 off-rate TTM/ligand pairs, analysis can be accomplished by
 11 simply removing the solution containing the labelled TTM.
 12 This can be done by centrifugation to remove unbound TTMs.
 13 Samples of HPQ ligand/125-I Streptavidin were assembled as
 14 in Example 16 and subjected to comparative unbound TTM
 15 removal Tests 15-17 below. The loadings were 50 nmoles in
 16 all three tests.

17
 18 **TABLE V COMPARATIVE REMOVAL TESTS**

| | | CPM | $\times 10^6$ |
|----|---|--------|---------------|
| | | Bound | Total |
| 21 | TEST 15 CENTRIFUGATION: 13,000 RPM for 3 MIN. | | |
| 22 | HPQ | 0.56 | 0.7 |
| 23 | Amino functionalized* | 0.01 | 0.7 |
| 24 | TEST 16 VACUUM WASH | | |
| 25 | HPQ | 0.35 | 0.7 |
| 26 | Amino functionalized* | 0.0038 | 0.7 |
| 27 | TEST 17 VORTEX WASH | | |
| 28 | HPQ | 0.31 | 0.7 |
| 29 | Amino functionalized* | 0.007 | 0.7 |
| 30 | * No ligand. | | |

31
 32 As shown by comparing the bound CPM for centrifugal
 33 removal vs. vacuum wash and vortex wash, the
 34 centrifugation gives higher count for identical loadings.
 35 This shows that centrifugation does not remove TTMs
 36 specifically bound to ligands, even if the ligand or TTM
 37 has a very fast off-rate. The bound counts on non-washed,
 38 centrifugation-removed excess TTM represents an assay of
 39 the equilibrium enrichment of labelled target bound to the

1 system of the invention can be used quantitatively; that
 2 is, count readings (intensity) can be quantitatively
 3 correlated to amounts of bound TTMs. The high slope and
 4 total amount of bound counts indicates high sensitivity of
 5 the method.

6 **Example 17. Fast Off-Rate Analyses.**

7 Over washing can remove specifically bound ligands if
 8 these ligands have a fast off-rate. The more extensive
 9 the washing the more signal is removed. In cases of fast
 10 off-rate TTM/ligand pairs, analysis can be accomplished by
 11 simply removing the solution containing the labelled TTM.
 12 This can be done by centrifugation to remove unbound TTMs.
 13 Samples of HPQ ligand/¹²⁵I Streptavidin were assembled as
 14 in Example 16 and subjected to comparative unbound TTM
 15 removal Tests 15-17 below. The loadings were 50 nmoles in
 16 all three tests.

17

18 **TABLE V COMPARATIVE REMOVAL TESTS**

| | <u>CPM</u> <u>x10⁶</u> | |
|---|-----------------------------------|--------------|
| | <u>Bound</u> | <u>Total</u> |
| 21 TEST 15 CENTRIFUGATION: 13,000 RPM for 3 MIN. | | |
| 22 HPQ | 0.56 | 0.7 |
| 23 Amino functionalized* | 0.01 | 0.7 |
| 24 TEST 16 VACUUM WASH | | |
| 25 HPQ | 0.35 | 0.7 |
| 26 Amino functionalized* | 0.0038 | 0.7 |
| 27 TEST 17 VORTEX WASH | | |
| 28 HPQ | 0.31 | 0.7 |
| 29 Amino functionalized* | 0.007 | 0.7 |

30 * No ligand.

31

32 As shown by comparing the bound CPM for centrifugal
 33 removal vs. vacuum wash and vortex wash, the
 34 centrifugation gives higher count for identical loadings.
 35 This shows that centrifugation does not remove TTMs
 36 specifically bound to ligands, even if the ligand or TTM
 37 has a very fast off-rate. The bound counts on non-washed,
 38 centrifugation-removed excess TTM represents an assay of
 39 the equilibrium enrichment of labelled target bound to the

CLAIMS

1. A ligand display system comprising in operative combination:

5 a) a biocompatible substrate having the property of being covalently bondable with a hydrophilic polar multi-functionalized polymer;

b) a hydrophilic polar multi-functionalized polymer chain;

10 c) said polymer is covalently anchored to said substrate in at least one attachment point spaced along said polymer chain to form a thin film three dimensional matrix layer above said substrate in an open, freely permeable configuration for access therinto of target molecules for affinity binding to preselected ligands covalently tethered to said polymer matrix.

2. A ligand display system as in Claim 1 wherein:

a) said polymer is covalently anchored to said substrate by spacer arm molecules.

3. A ligand display system as in Claim 1 wherein:

5 a) said substrate is selected from the group consisting essentially of functionalized plastic, glass, alumina-containing material, silica-containing material, silicon-containing material, and combinations thereof.

4. A ligand display system as in Claim 3 wherein:

a) said functionalized plastic substrate is a polyolefin plastic.

5. A ligand display system as in Claim 4 wherein:

a) said polyolefin plastic is porous, and is in the form of a thin disc functionalized for attachment to said polymer.

6. A ligand display system as in Claim 1 wherein:

a) said polymer is a polysaccharide having $[S]_n$

saccharide units, wherein n ranges from about 100 to about 50,000.

7. A ligand display system as in Claim 6 wherein:

a) said polysaccharide is selected from the group consisting essentially of dextran, carboxymethylcellulose, guaicaic acid, copolymers and mixtures thereof having a molecular weight in the range of from about 10,000 to about 10 million.

8. A ligand display system as in Claim 7 wherein:

a) said polysaccharide includes functional groups, FG, with the number of functional groups FG per saccharide unit [S] in the range of from about 1:1 FG:[S] to about 1:100 FG:[S].

9. A ligand display system as in Claim 1 which includes:

a) preselected ligands covalently tethered to said polymer.

10. A ligand display system as in Claim 9 wherein:

a) said ligands are mono-tethered to said polymer.

11. A ligand display system as in Claim 9 wherein:

a) said ligands are constructed in situ on said matrix by chemical assembly of synthons.

12. A ligand display system as in Claim 11 wherein:

a) the loading of said ligands on said polymer ranges from about 1 ligand per monomeric unit of said polymer to about 1 ligand per 100 monomeric units of said polymer.

13. A ligand display system as in Claim 12 wherein:

a) said ligand is a MER_n of the same or different monomeric units, and n ranges from about 2 to about 100.

14. A ligand display system as in Claim 13 wherein:

a) at least some of said ligands comprise a preselected library of related compounds.

15. A ligand display system as in Claim 14 wherein:
a) said library is a peptide library.

16. A ligand display system as in Claim 9 which includes:
a) at least one preselected target molecule affinity bonded to at least one of said ligand molecules.

17. A ligand display system as in Claim 16 wherein:
a) said target molecule has therapeutic drug properties, and is slowly hydrolyzable from said ligand to provide slow release drug delivery in contact with a body tissue or fluid.

18. A method of drug delivery comprising the steps in operative sequence of:

- a) providing a ligand display system as in Claim 17; and
b) contacting said polymer matrix having said target molecule affinity bound thereto with a tissue or body fluid for a time sufficient to permit release from said polymer matrix of said target molecule drug for ameliorative effect.

19. A method of ligand/target molecule affinity binding analysis comprising the steps in operative sequence of:

- a) providing a ligand display system as in Claim 1;
b) covalently tethering preselected ligand molecules to said polymer for display;
c) contacting said displayed ligands with a solution containing a selected sample having target molecules therein;
d) maintaining said contact under conditions and for a time sufficient for potential affinity bonding to occur;
e) removing excess unbound target molecules; and
f) detecting affinity bound ligand/target molecule conjugates.

20. An affinity analysis as in Claim 19 wherein:

- a) said ligands are a library of related MER_n ligands.

21. An affinity analysis as in Claim 20 wherein:

5 a) the ligand library MERs are peptides constructed from amino acids forming an n-peptide library.

22. An affinity analysis as in Claim 20 wherein:

a) said target molecules are tagged.

23. An affinity analysis as in Claim 22 which includes the added steps of:

a) recovering at least some of the affinity bound ligands and the target molecule conjugates thereof; and

5 b) determining the structure of at least one of said ligands or said target molecules of at least one ligand/target molecule conjugate.

24. An affinity analysis as in Claim 19 which includes the added step of:

a) constructing ligands from synthons covalently bonded to said polymer.

25. A method of preparing a ligand display system comprising the steps in operative sequence of:

5 a) functionalizing a biocompatible substrate with functional groups for covalent anchoring thereto of a hydrophilic polar multi-functionalized polymer chain thereto;

10 b) anchoring said polymer to said substrate in a plurality of attachment points spaced along said polymer chain to form a three dimensional matrix layer above said matrix in an open, freely permeable configuration; and

c) forming tethers on said polymer for covalent bonding thereto of ligands for display for affinity screening of preselected target molecules.

26. A method of ligand display system preparation as in Claim 25 wherein:

5 a) said anchoring step includes use of intermediate anchor molecules to staple said polymer to said substrate in said plurality of attachment points spaced along said

polymer chain.

27. A method of ligand display system preparation as in Claim 26 wherein:

5 a) said anchor molecules are reacted with functional groups on said substrate to covalently bond said anchor molecules adjacent one end thereof and leave a free end for reaction with said polymer;

b) said polymer is functionalized with functional groups, FG, for reaction with said anchor molecule free end; and

10 c) at least some of the functionalized groups on said polymer are reacted with said anchor molecules free ends.

28. A method of ligand display system preparation as in Claim 27 wherein:

a) said anchor molecules free ends are functionalized before reaction with said polymer.

29. A method of ligand display system preparation as in Claim 25 wherein:

5 a) said polymer functionalizing step includes multi-functionalizing said polymer for reaction both to form said anchors and to react with tethers for said ligands.

30. A method of ligand display system preparation as in Claim 26 wherein:

5 a) said polymer functionalizing step includes multi-functionalizing said polymer for reaction both with said anchor molecules and to react with tethers for said ligands.

31. A method of ligand display system preparation as in Claim 30 wherein:

5 a) said polymer is a polysaccharide having $[S]_n$ saccharide units, wherein n ranges from about 100 to about 50,000.

32. A method of ligand display system preparation as in

Claim 31 wherein:

5 a) said polysaccharide is selected from the group consisting essentially of dextran, carboxymethylcellulose, guaicaic acid, copolymers and mixtures thereof having a molecular weight in the range of from about 10,000 to about 10 million.

33. A method of ligand display system preparation as in Claim 32 wherein:

5 a) said polysaccharide includes functional groups, FG, with the number of functional groups FG per saccharide unit [S] in the range of from about 1:1 FG:[S] to about 1:100 FG:[S].

34. A method of ligand display system preparation as in Claim 33 includes the added step of:

a) covalently bonding ligands to said tethers.

35. A method of ligand display system preparation as in Claim 34 wherein said step of bonding ligands to tethers includes:

a) constructing in situ, ligands by synthesis from synthons, which ligands are mono-tethered to said HPMP matrix.

36. A method of ligand display system preparation as in Claim 35 wherein:

a) the loading of said ligands on said polymer ranges from about 1 ligand per polysaccharide unit [S] to about 1 ligand per 100 polysaccharide unit [S].

37. A method of ligand display system preparation as in Claim 36 wherein:

a) said ligand is a MER_n of the same or different monomeric units, and n ranges from about 2 to about 100.

38. A method of ligand display system preparation as in Claim 37 wherein:

a) at least some of said ligands comprise a preselected

library of related compounds.

39. A method of ligand display system preparation as in Claim 38 wherein:

a) said library is a peptide library.

40. A method of ligand display system preparation as in Claim 27 wherein:

a) said polymer matrix forms a layer in the range of from about 100 - 2000 Å in thickness;

5 b) said polymer has a molecular weight in the range of from about 10,000 to about 10 million;

c) the anchor density is in the range of from 0.001% to 25% of available substrate surface functionalized sites; and

10 d) the anchors and tethers are selected from C₂-C₃₀ alkanes, polyethers and combinations thereof.

AMENDED CLAIMS

[received by the International Bureau
on 01 August 1994 (01.08.94);
original claims 1 and 25 amended; remaining claims unchanged
(4 pages)]

1. A ligand display system for affinity binding comprising in operative combination:

5 a) a biocompatible essentially monophasic substrate having the property of being covalently amide bondable with a hydrophilic polar multi-functionalized polymer, said bond permitting chemically mediated peptide assembly on said polymer without substantial cleavage of said amide bond;

b) a hydrophilic polar multi-functionalized polymer chain;

10 c) said polymer is covalently, substantially permanently anchored to said substrate in at least one attachment point spaced along said polymer chain to form a thin film three dimensional, substantially non-crosslinked matrix layer above said substrate in an open, freely permeable configuration for access therinto of target molecules for affinity binding to preselected ligands covalently, substantially permanently tethered to said polymer matrix for affinity screening of target molecules not involving evanescent field techniques.

2. A ligand display system as in Claim 1 wherein:

a) said polymer is covalently anchored to said substrate by spacer arm molecules.

3. A ligand display system as in Claim 1 wherein:

a) said substrate is selected from the group consisting essentially of functionalized plastic, glass, alumina-containing material, silica-containing material, silicon-containing material, and combinations thereof.

4. A ligand display system as in Claim 3 wherein:

a) said functionalized plastic substrate is a polyolefin plastic.

5. A ligand display system as in Claim 4 wherein:

a) said polyolefin plastic is porous, and is in the form of a thin disc functionalized for attachment to said polymer.

6. A ligand display system as in Claim 1 wherein:

a) said polymer is a polysaccharide having $[S]_n$

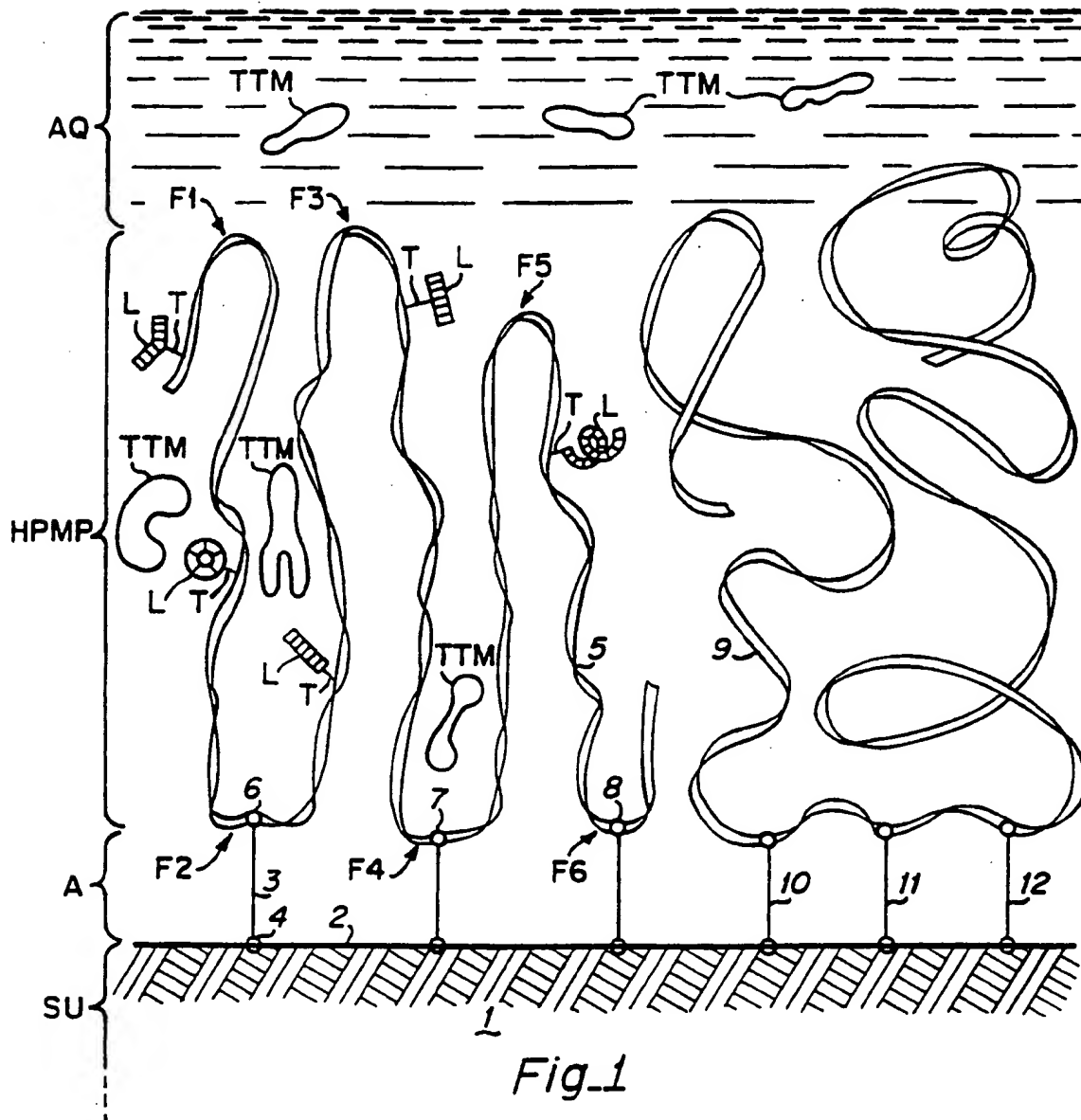
21. An affinity analysis as in Claim 20 wherein:
- a) the ligand library MERs are peptides constructed from amino acids forming an n-peptide library.
22. An affinity analysis as in Claim 20 wherein:
- a) said target molecules are tagged.
23. An affinity analysis as in Claim 22 which includes the added steps of:
- a) recovering at least some of the affinity bound ligands and the target molecule conjugates thereof; and
 - b) determining the structure of at least one of said ligands or said target molecules of at least one ligand/target molecule conjugate.
24. An affinity analysis as in Claim 19 which includes the added step of:
- a) constructing ligands from synthons covalently bonded to said polymer.
25. A method of preparing a ligand display system comprising the steps in operative sequence of:
- a) functionalizing a biocompatible, essentially monophasic substrate with functional groups for amide covalent bond anchoring thereto of a hydrophilic polar multi-functionalized polymer chain;
 - b) anchoring said polymer to said substrate in a plurality of attachment points spaced along said polymer chain to form a three dimensional, substantially non-crosslinked matrix layer above said substrate in an open, freely permeable configuration; and
 - c) forming tethers on said polymer for covalent bonding thereto of ligands for display for affinity screening of preselected target molecules not involving evanescent field techniques.
26. A method of ligand display system preparation as in Claim 25 wherein:

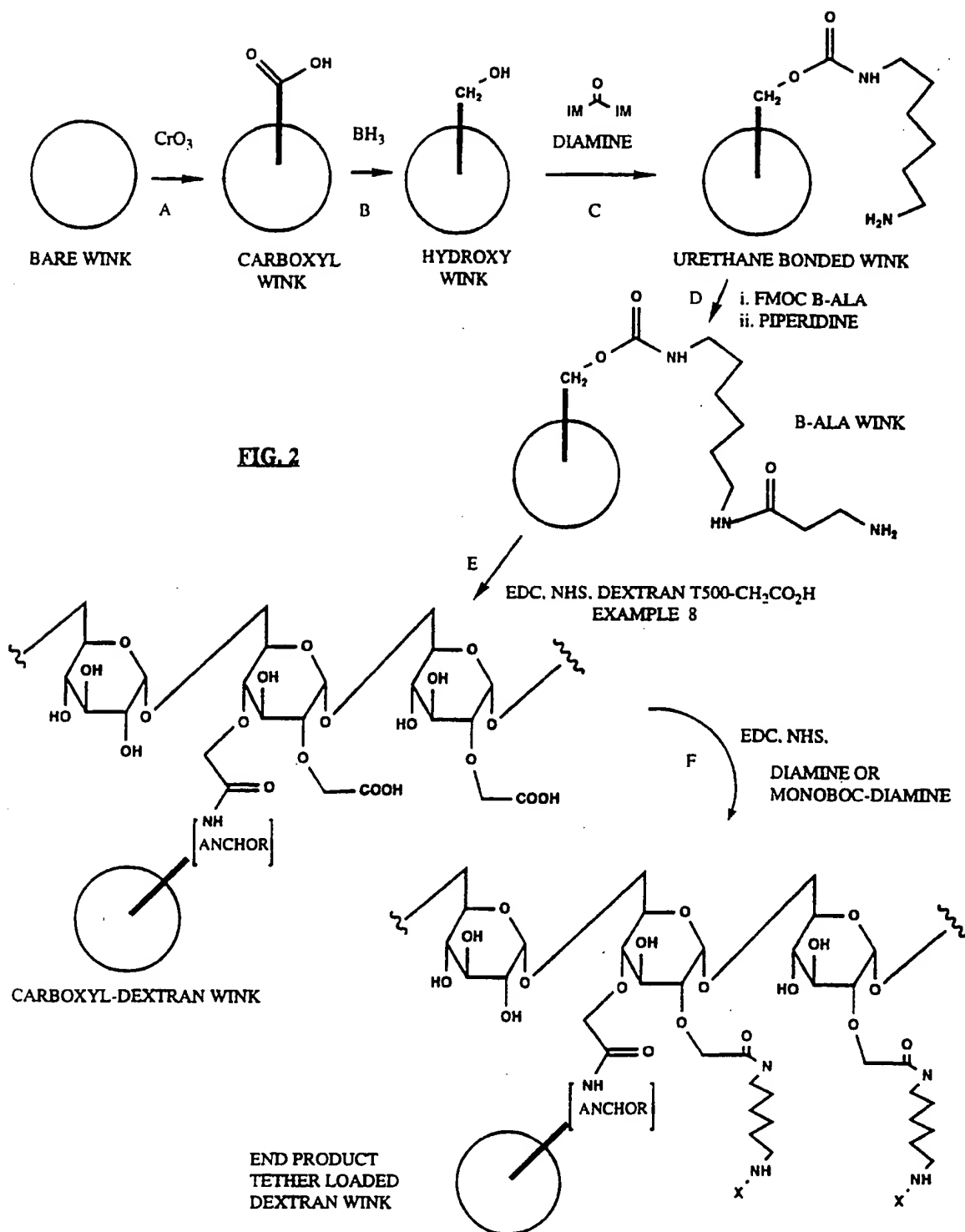
a) said anchoring step includes use of intermediate anchor molecules to staple said polymer to said substrate in said plurality of attachment points spaced along said polymer chain.

STATEMENT UNDER ARTICLE 19

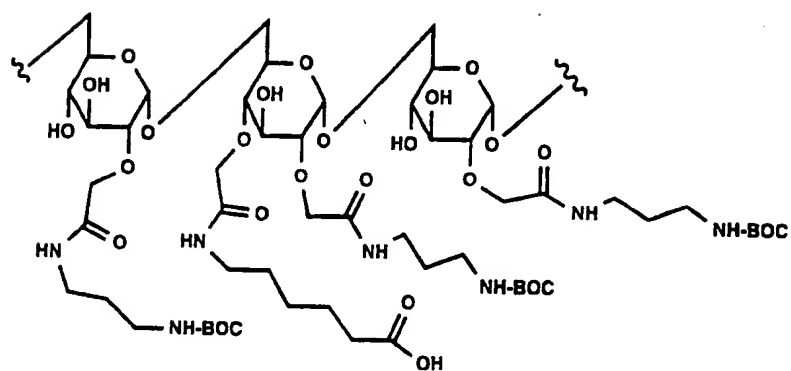
No new matter is introduced by this Amendment. The amended claims do not affect either the description or the drawings. Additions to the claims are underscored and deleted words are bracketed. In accordance with PCT Rule 26.4 replacement sheets comprising pages 33, 33a, 36 and 36a corresponding to the modified pages 33 and 36 of the International Application as originally filed and containing amended claims 1 and 25 are attached hereto.

Applicant submits this amendment to distinguish this invention from the inventions and methods disclosed in the International Search Report with the intent to facilitate the International Preliminary Examination and the individual examinations during the National Phase. More particularly, the main claims 1 and 25 in calling for the substrate as monophasic and the affinity binding as not involving evanescent field techniques clearly distinguishes the Buckle et al frustrated total reflectance (FTR) and the surface plasma resonance (SPR) techniques. FTR relies on an oxide formed on the substrate layer to provide a dielectric layer, whereas there is no such reliance in Applicant's invention.





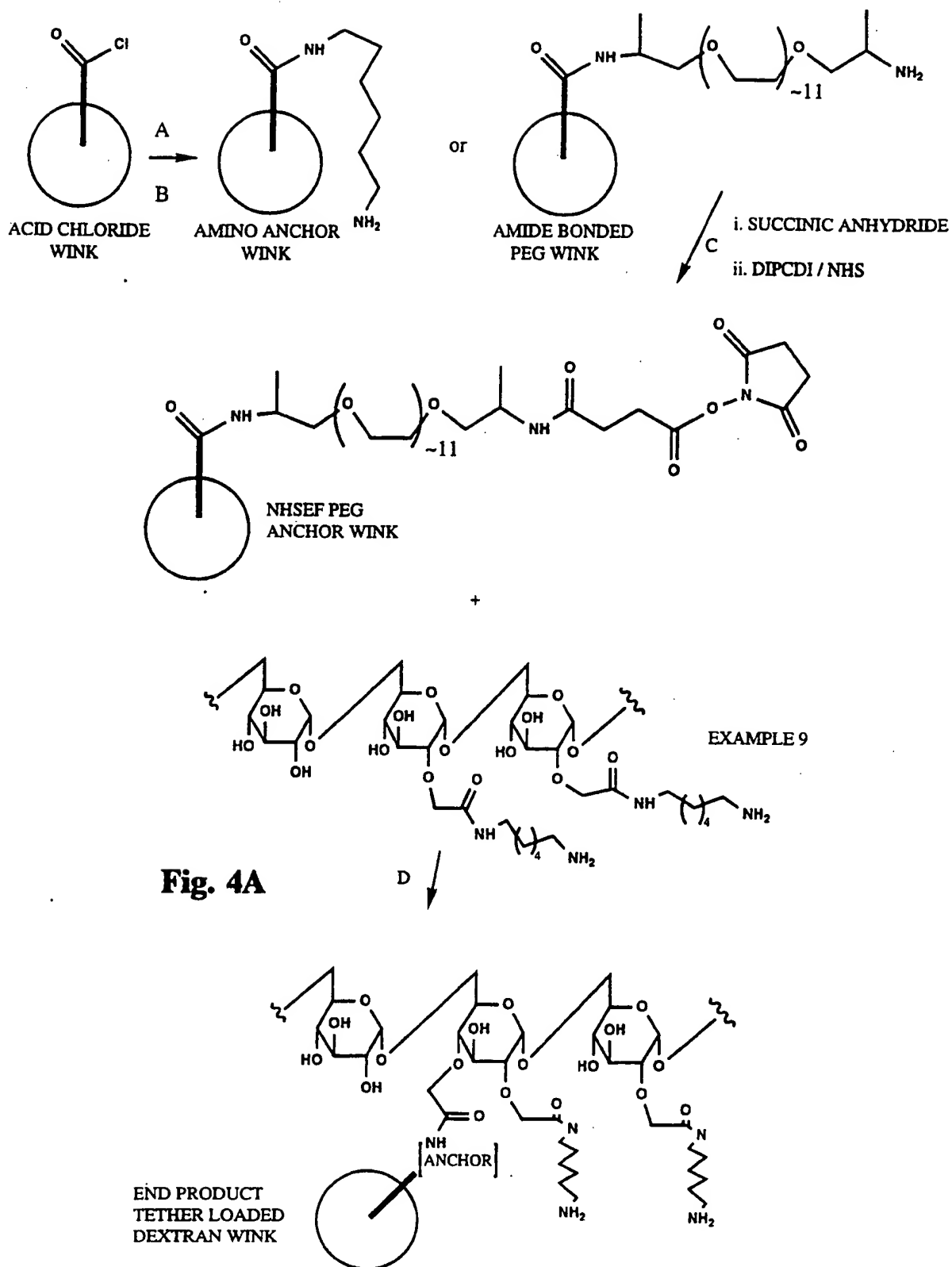
The chemical structure shows a branched poly(amide amine) dendron. It consists of three repeating units of 1,3-bis(2-aminoethyl)urea linked by urea bonds. Each terminal amino group is protected with a BOC (tert-butoxycarbonyl) group. The structure is terminated with a methyl ester group (OCH₃) and a methyl group (CH₃).



The diagram illustrates a branched dendritic polymer structure. It features a central core, represented by a circle with a diagonal line, labeled "NH ANCHOR". This core is connected to a network of amide bonds. The structure branches out into three main arms, each terminating in a primary amine group (NH_2). The polymer also includes several hydroxyl (OH) groups and ether linkages, suggesting a complex, multi-functional architecture. The overall structure is symmetrical and highly branched, characteristic of dendritic polymers used in various applications like catalysis or materials science.

SUBSTITUTE SHEET (RULE 26)

4/12



5/12

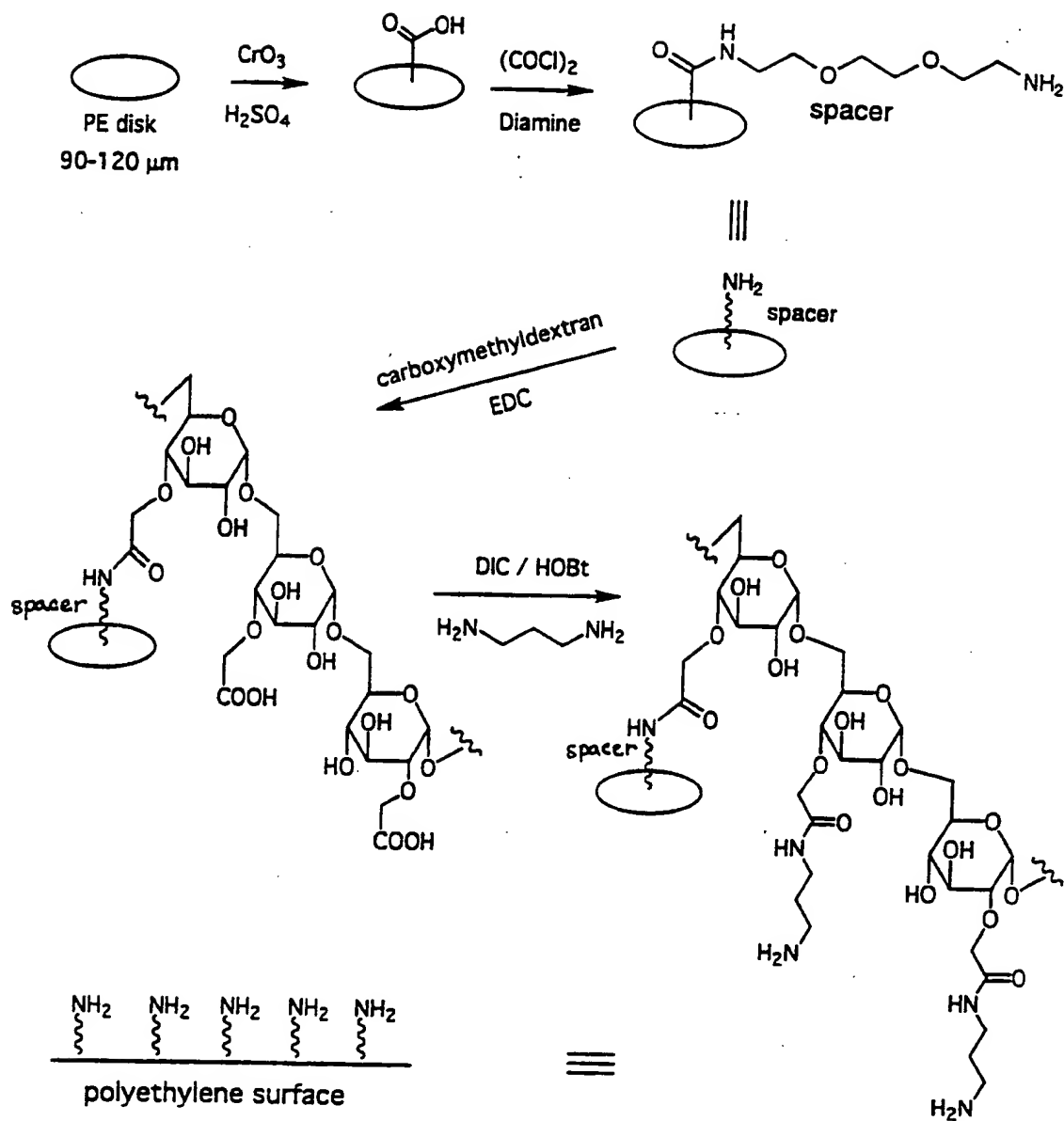


Fig. 4B

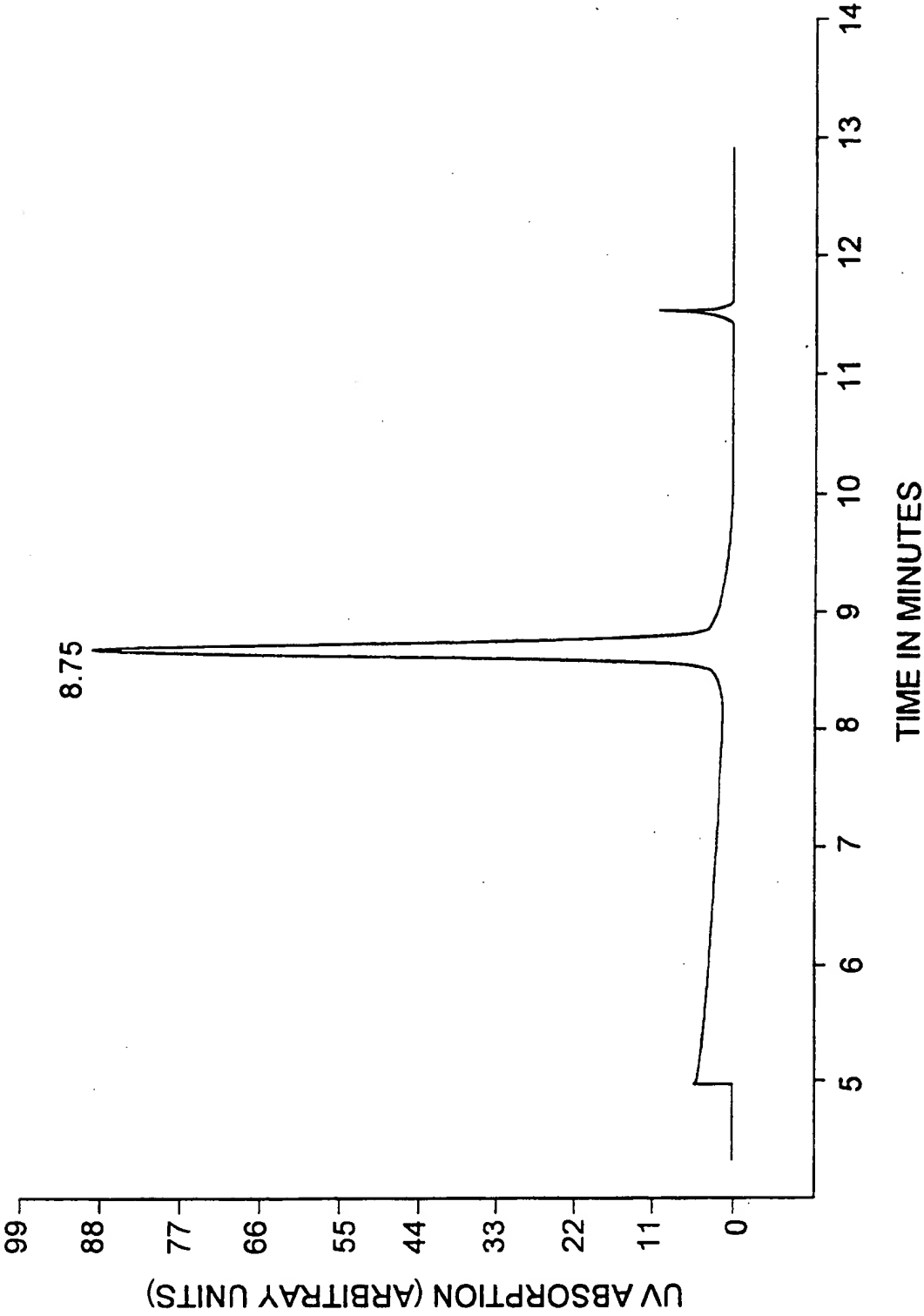


FIG. 5

7/12

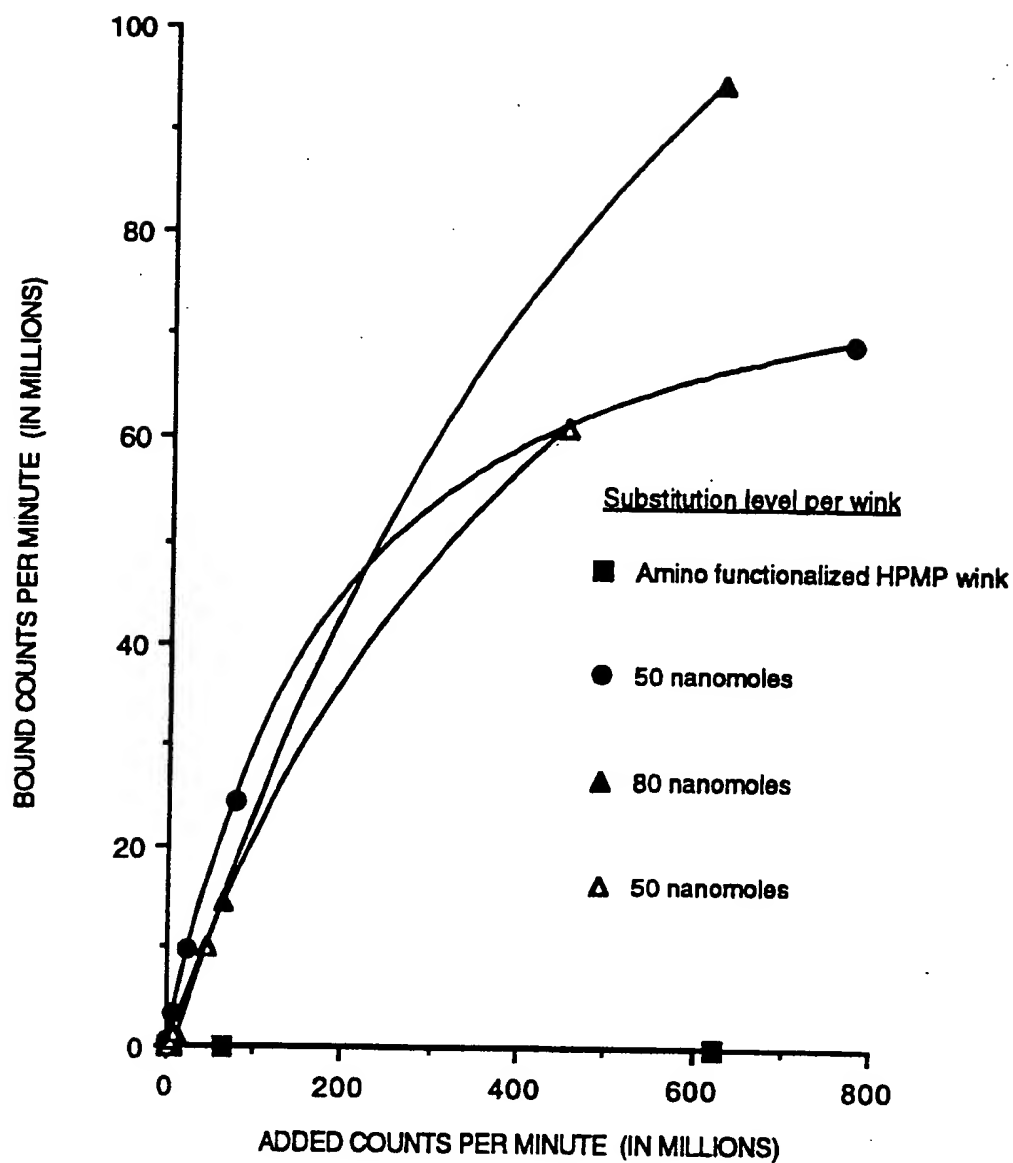
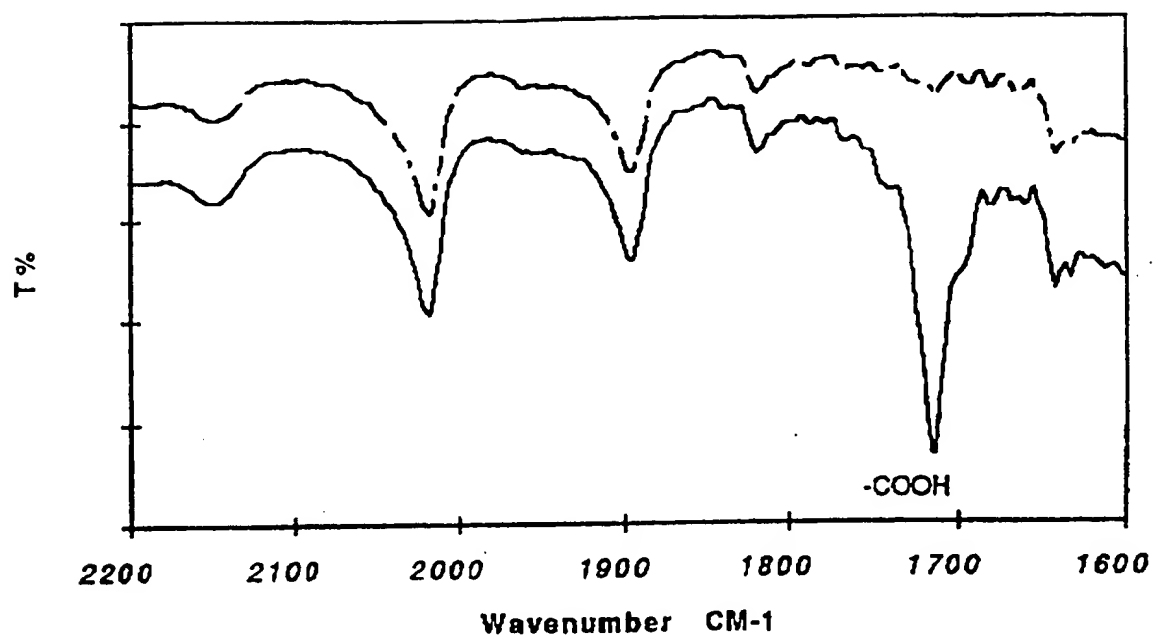
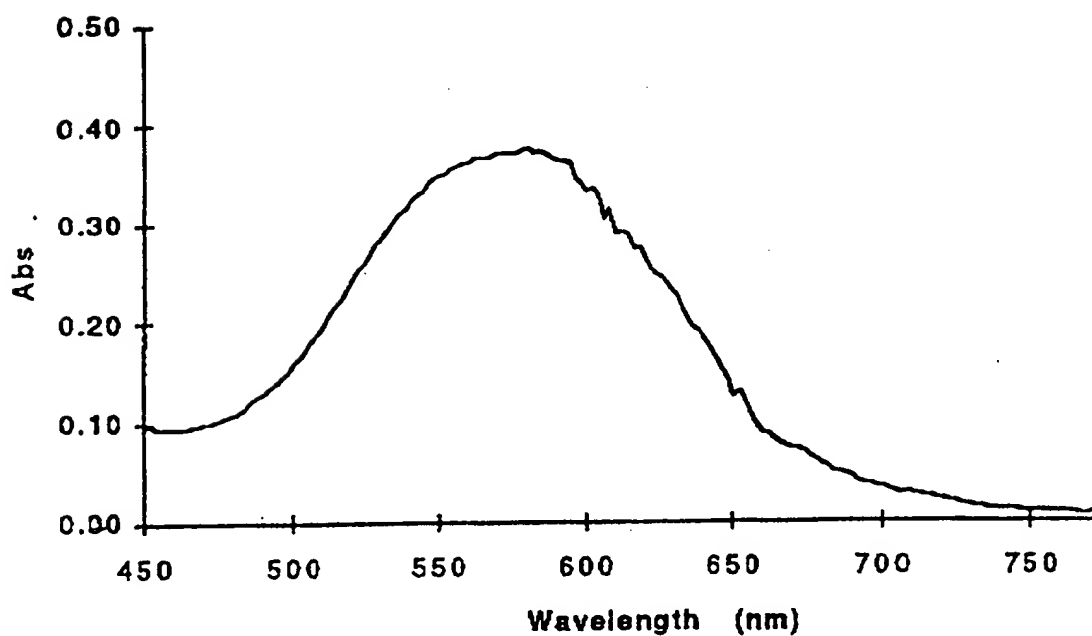
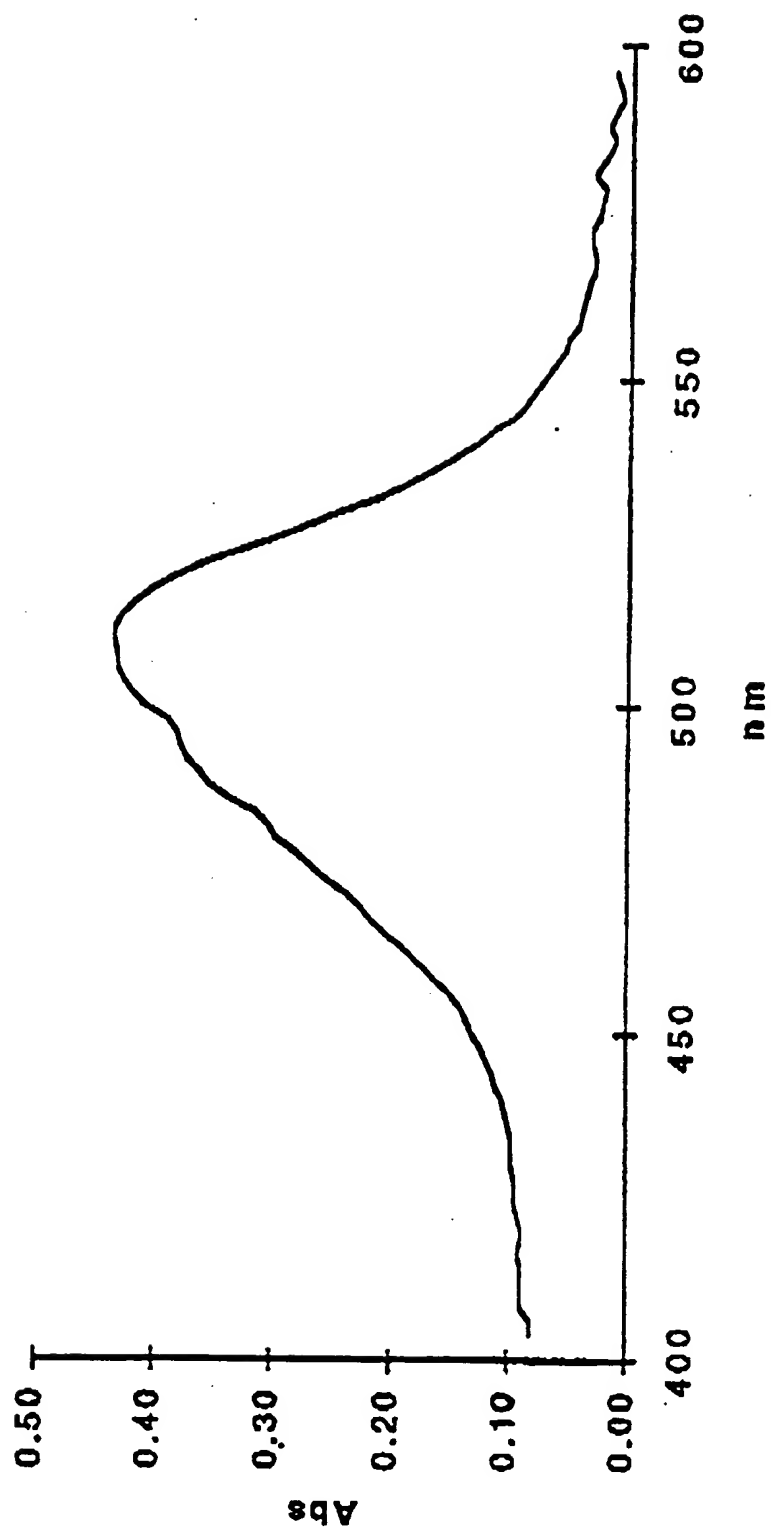


Fig. 6

*Fig. 7**Fig. 8*

*Fig. 9*

10/12

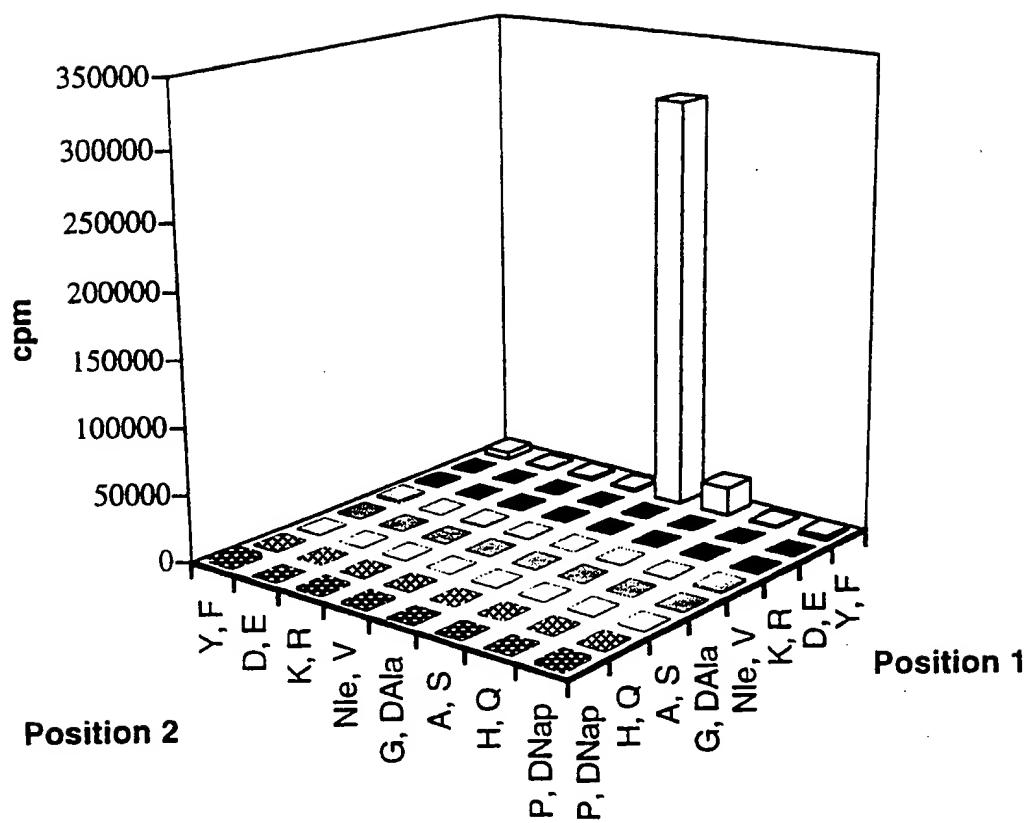


Figure 10

11/12

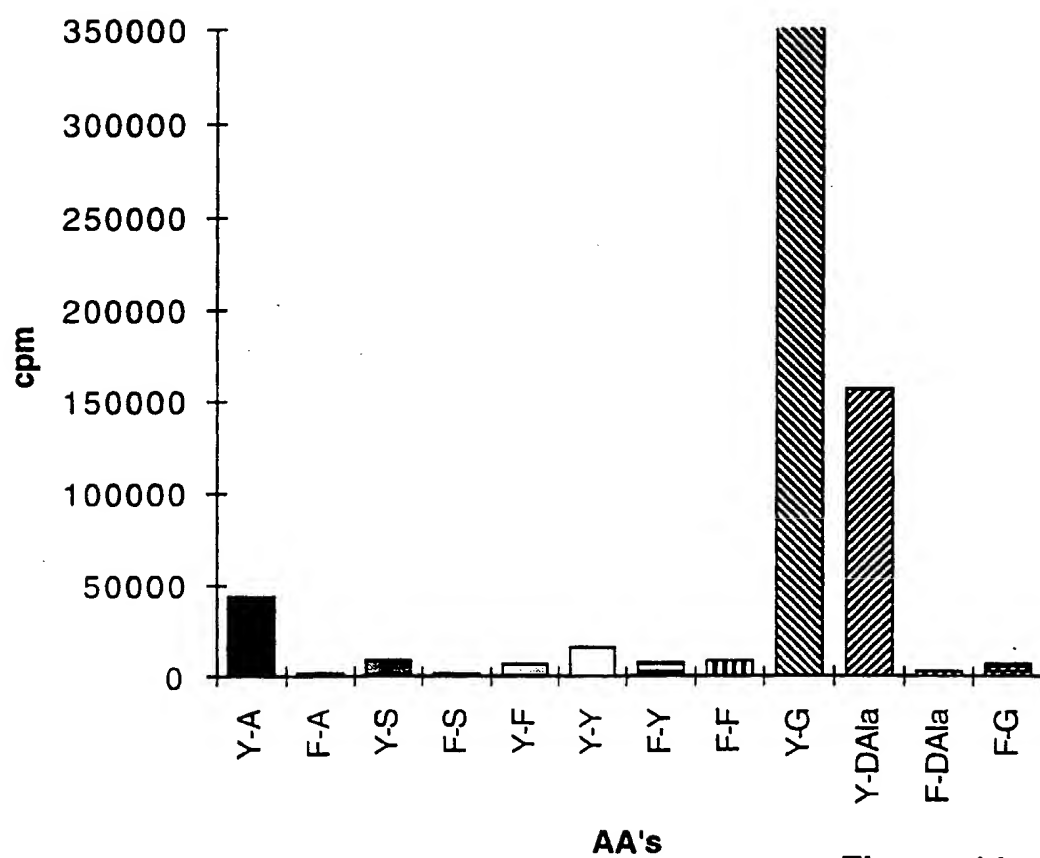


Figure 11

12/12

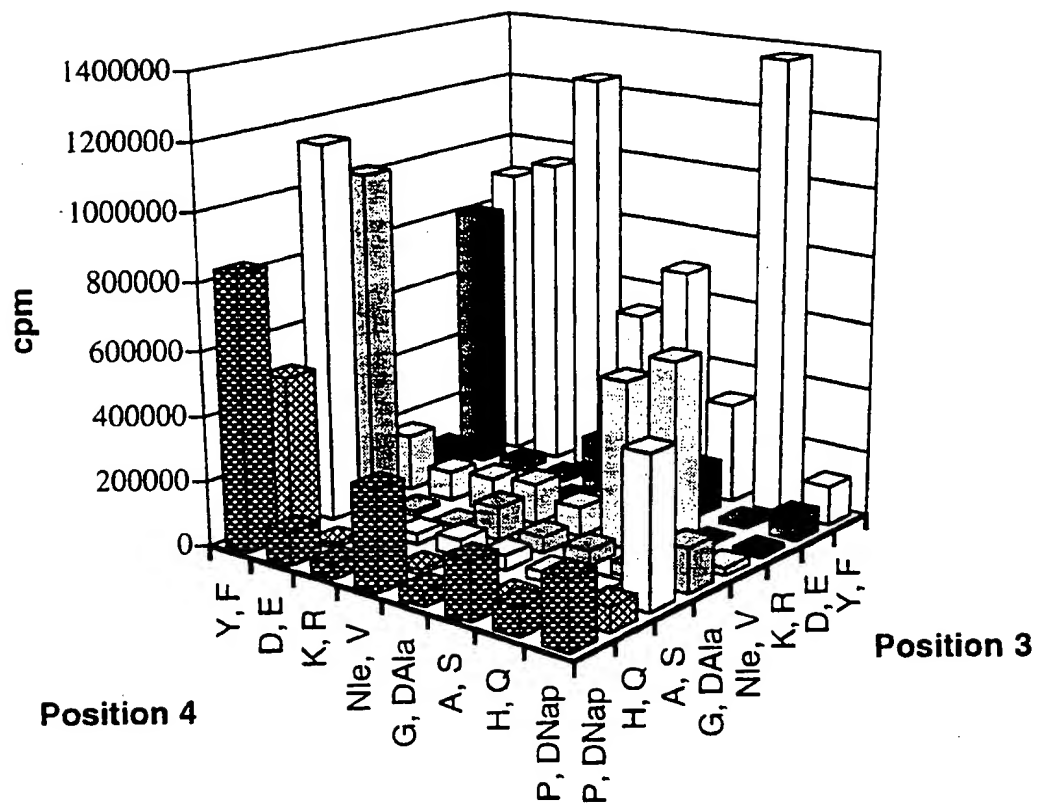


Figure 12

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : G01N 33/548; B05D 3/10

US CL : 436/530; 428/532; 427/300; 424/425

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/530, 501, 518, 528, 529, 532; 428/532; 427/300, 415; 530/334; 424/425

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---------------|---|---|
| X --- Y | WO, A, 92/21976 (BUCKLE ET AL) 10 December 1992, see page 2, paragraph bridging pages 4-5, page 6, pages 10 and 11. | 1-10, 12, 13, 16, 19, 25-37, 40 ----- 11, 14, 15, 20-24, 38, 39 |
| Y | WO, A, 90/05303 (BERGSTROM ET AL) 17 May 1990, see pages 8-12. | 1-40 |
| Y | US, A, 4,822,681 (SCHOSSLER ET AL.) 18 April 1989, see column 4, lines 47-62. | 1-40 |

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

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|---|--|
| Date of the actual completion of the international search 21 MAY 1994 | Date of mailing of the international search report 01 JUN 1994 |
| Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230 | Authorized officer Lora M. Green <i>Jill Warden for</i> Telephone No. (703) 308-0196 |

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|---------------------------|
| Y | Current Opinion in Biotechnology, Vol. 3, issued 1992, Birnbaum et al., "Peptide Screening", pages 49-54, see abstract and pages 50-52. | 11, 14, 15, 20-24, 38, 39 |
| Y | International Journal of Pharmaceuticals, Vol. 73, issued 1991, Wan et al., "The effect of hydroxypropylmethylcellulose on water penetration into a matrix system", pages 111-116, see Introduction. | 17, 18 |

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